IMMUNOCHEMISTRY OF *FASCIOLA HEPATICA* IN THE RAT MODEL

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DECLARATION

I hereby declare that the work presented in this thesis is the product of my own efforts except where specifically stated in the text and in the acknowledgement section. The work was carried out in the Centre for Tropical Veterinary Medicine, University of Edinburgh under the supervision of Dr. Leslie Harrison and Professor M.M.H. Sewell. No part of this thesis has been submitted in any previous application for a degree.

J.O. Ajànusi

This thesis is dedicated to:

My late daughter Beatrice Ebenmosi (02/09/92-27/09/92)

My wife Justina

My parents, Chief and Mrs Isa Ajanusi.

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LIST OF ABBREVIATIONS

°C	=	degrees Centigrade
g	=	relative centrifugal force
min	=	minute
PCV	=	Packed Cell Volume
Kg	=	Kilogram
cm^2	=	Square centimetre
mm	=	millimetre
ml	=	millilitre
µm	=	micrometre
µg	=	microgram
µl	=	microlitre
v/v	=	volume for volume
w/v	=	weight for volume
$NaHCO_3$	=	Sodium bicarbonate
NaCl	=	Sodium chloride
$MgCl_2$	=	Magnesium chloride
PBS	=	Phosphate buffered saline
H & E	=	Haematoxylin and Eosin
PBS-PI	=	PBS containing protease inhibitors
CTAB	=	Cetyl Trimethyl Ammonium Bromide
NoG	=	N-octyl glucopyranoside
D0	=	Day 0
D1	=	Day 1
D14	=	Day 14
D28	=	Day 28
D42	=	Day 42
D56	=	Day 56
ES	=	Excretory/Secretory products
ES_0	=	ES of D0 flukes
ES_1	=	ES of D1 flukes
ES_{14}	=	ES of D14 flukes
ES_A	=	ES of adult flukes
mMol	=	millimolar
MBq	=	megabecquerel
µci	=	microcurie
NP40	=	nonidet P40
ELISA	=	Enzyme Linked Immunosorbent Assay
NGS	=	Normal Goat Serum
IgG	=	Immunoglobulin
mA	=	milliamp
EDTA	=	Ethylene Diamine Tetra Acetic Acid
kDa	=	Kilodalton
NC	=	Nitrocellulose membrane
SDS-PAGE	=	Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis
+NRS	=	given normal rat serum
+IRS	=	given immune rat serum
NS	=	given no serum
PVG	=	Piebald Viral Glaxo

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SUMMARY

The economic impact of fasciolosis on livestock industries including those in tropical countries such as Nigeria has prompted research into various aspects of the host-parasite relationship in fasciolosis. An improved knowledge of this interaction should contribute to the development of control measures for fasciolosis. The excretions, secretions and surface components of a parasite are by their nature centrally involved in the host/parasite interaction. Rats, like cattle, are capable of developing resistance to fasciolosis after primary infection and are therefore considered a suitable laboratory model for cattle. The objective of this study was to characterise the excretory/secretory (ES) and surface components of *Fasciola hepatica* as it develops in the rat, and to identify those components involved in the host/parasite interaction that may have diagnostic and/or protective value.

In the first instance the available scientific literature on the immunochemical nature of *Fasciola* spp. and on natural and experimentally induced resistance to *F. hepatica* infection in various hosts was reviewed.

Three trials were conducted during the study in order to produce supplies of rat antiserum which was protective against *F. hepatica*. Rats were infected with either 10 (first trial) or 20 (second and third trials) *F. hepatica* metacercariae as information from the literature indicated that these doses were adequate to stimulate the production of protective antibody levels.

The rat sera from the three trials were checked for the presence of protective antibodies by passive protection studies. Only in the latter two trials was the level of protection conferred on recipient statistically significant. The probable causes for the lack of significant protection in trial 1 are discussed.

The silver stained protein profiles of ES from newly excysted (D0) flukes and one-day old (D1) flukes were characteristic and were similar to each other. The ES of parenchymal 14-day old (D14) and adult (D56) flukes were markedly different from D0 and D1 flukes but similar to each other. The silver stained total

ES protein profiles of the developing flukes were very different from the total biosynthetically (^{35}S-methionine) radio-labelled ES protein profiles. Possible reasons for this are discussed. However, as with the total silver stained ES protein profiles there were clear changes in the profiles of the biosynthetically radio-labelled ES as the flukes developed. It is suggested that these differences in the ES products may reflect the changing environment and activities of the flukes. The possible functions of the changing ES products are discussed.

Western blot studies of adult fluke ES using sera collected sequentially from rats infected with *F. hepatica* in the three trials revealed a 25 kDa doublet (reduced) and 26 and 27 kDa antigens (non-reduced). It is thought possible that these antigens provoke a protective antibody response. The rat sera (I_2) used for the second protection trial contained more antibodies which reacted with these particular antigens than the sera used in the other 2 trials and had a demonstrably greater protective ability. The 25 kDa doublet was also identified in D14 ES but not in the D0 or D1 ES. In D0 and D1 ES, however, an immunodominant reduction-sensitive 191kDa antigen was identified.

Western blot studies on D0, D14 and adult ES using sequential bleeds from rats injected with the protective I_2 serum prior to challenge infection revealed two interesting points. Firstly, in rats that were completely protected from infection antibody activity against the 25, 26 and 27 kDa antigens in D14 and adult fluke ES and against the 191 kDa antigen in D0 and D1 ES gradually declined as the experiment progressed. Secondly, in partially protected rats although the response to these antigens had also declined by D14 and D28, it then rose again by D42 and D56. Thus, although these antigens may be involved in promoting a protective immune response, the antibodies against them are also good indicators of infection.

Immune-coprecipitation of biosynthetically radio-labelled D0 ES with sera from infected rats in trial 2 revealed a 26 kDa antigen. It is suggested that this antigen may be the same as the 25 kDa antigen detected in western blots of D14 and

adult fluke ES and that it was not detected in Western blots of D0 and D1 ES due to the limited assay sensitivity. A novel antigen of about 38kDa was strongly detected by serum I_2 containing protective antibody in immune-coprecipitation of biosynthetically radio-labelled ES of parenchymal (D14, D28 and D42) flukes. It is suggested that this antigen may also have protective and/or diagnostic properties.

The study thus indicated that the following *F. hepatica* antigens merit further study; the 191kDa antigen in D0 ES, the 25 kDa in D14 ES and the 25, 26 and 27 kDa antigens in adult ES, in order to further elucidate their role in the host/parasite relationship. Similarly, the 26 kDa antigen detected in immuno-coprecipitation of biosynthetically (^{35}S-methionine) radio-labelled D0 ES, and the 38 kDa antigen detected in D14, D28 and D42 ES deserve further investigation. The fact that these antigens were being actively synthesized by the parasite at the time of study suggests that it would be feasible to clone them.

In conclusion, the possible relevance of the above antigens for diagnosis and protection in fasciolosis is discussed, as is the potential use of non-immunogenic biosynthetically radio-labelled ES components in diagnostic assays designed to detect parasite products. Some components of this nature were detected in the course of this study.

Overall, the study has clearly identified antigenic components in the ES and surface extracts of *F. hepatica* which are involved in the host/parasite interaction in the rat model and, by implication, may also be of significance in bovine fasciolosis. The antigens detected by western blotting and by immuno-coprecipitation of D0 ES (that is those of 191 and 26 kDa) as well as the 38 kDa antigen detected in the ES from parenchymal flukes have not been previously reported in the rat model.

CHAPTER ONE

INTRODUCTION

INTRODUCTION

Fasciolosis is caused by several species of digenetic trematodes belonging to the genera *Fasciola* and Fascioloides (Soulsby, 1982).

The two known *Fasciola* spp. which are parasitic in domestic animals and sometimes in man are *F. hepatica* (Linnaeus 1758) and *F. gigantica* (Cobbold, 1885) (cited in Soulsby, 1982). *F. hepatica* occurs in the temperate regions (Hunter and Health, 1984; Lemma, Gabre and Telda, 1985; Sharma, Dhar, and Raina, 1989), while *F. gigantica* occurs in the tropical regions particularly in Africa, Indian subcontinent, parts of China, South East Asia, Philippines and Hawaii (Boray, 1969; Mzembe and Chaudhry, 1980; Soulsby, 1982; Lemma, *et al.*, 1985; Sharma *et al.*, 1989). Overlapping infection with both species occur in some parts of Africa and the Indian subcontinent (Kendall, 1954; Guralp, Ozcan and Simms, 1964; Boray, 1969).

The major hosts of these parasites are sheep, goats, cattle and buffalo. The horse, donkey, mule and camel are less susceptible but epidemiologically important while the domestic pig is naturally resistant to infection (Soulsby, 1982; Boray, 1969). Wild mammals may also contract fasciolosis and some of these act as reservoir hosts (Hammond, 1972).

Estimates of the prevalence rates of fasciolosis in tropical livestock are largely conjectural, although available figures suggest prevalence rates of between 30% and 90% are common in the highly endemic areas of East, West and Central Africa (Ogambo-Ongoma, 1972; Diaw, Seya and Sarr, 1988; Babalola and Schillhorn, 1976). The situation appears to be similar in tropical Asia, particularly in some parts of Bangladesh (Rahman, Ali and Rahman, 1972) and India (Sharma, *et al.*, 1989).

The economic losses caused by fasciolosis can be very serious, particularly in areas where the local environmental conditions are favourable to the intermediate

hosts, so that the final hosts are exposed to a high rate of infection which may lead to acute fasciolosis with both high morbidity and mortality (Sewell, 1976).

In such areas susceptible animals are often exposed to large doses of infection resulting in acute form of the disease, which is often associated with traumatic hepatitis inflicted by migrating immature flukes in the parenchyma (Soulsby, 1982). This acute form of the disease is often associated with high mortalities particularly in small ruminants (Henderson, 1936; Gretillat, 1961; Schillhorn Van Veen, 1979; Karib, 1962).

The chronic form of the disease, which commences when the fluke enters the bile duct, is responsible for heavy direct and indirect losses, especially in cattle (Chick, 1979). This is probably why Fabiyi (1986) described fasciolosis as the most important single helminth infection of cattle. The direct loss is brought about by liver condemnation (Fabiyi and Adeleye, 1982) while the indirect loss is caused by the reduced growth rate arising from increased protein catabolism coupled with reduced feed intake (Sewell, 1966; Dargie, Berry and Parkins, 1979) and lower feed conversion efficiency (Chick, Coverdale and Jackson, 1980). These losses are aggravated when only a low plane of nutrition is available for the animal as often occurs during the hot dry season in the tropics (Webster and Wilson, 1980) and in the autumn in temperate countries (Roseby, 1970; Reid, Doyle Armour and Jennings, 1972). Other indirect losses during the chronic phase of fasciolosis include a reduction in the yield and quality of milk (Froyd, 1978), a decrease in the number and activity of spermatozoites (Ramaniuk, 1978) and reduced wool growth in sheep (Hawkins and Morris, 1978).

The control of fasciolosis involves management procedures and the use of anthelmintics and molluscicides. These are expensive and not often available. For example pasture management or controlled grazing procedures, which may be used as control measures against *Fasciola* infection, do require additional labour and

fencing (Sewell, 1976) while the repeated use of anthelmintic or molluscicides is expensive.

Some hosts such as rats (Hayes, Bailer and Mitrovic, 1972, 1974a; Haroun, Hammond and Sewell, 1980a) and cattle (Ross, 1966; Doyle, 1971) develop significant resistance to fasciolosis following a primary infection. Several workers (Armour and Dargie, 1974; Hayes, Bailer and Mitrovic, 1974b; Haroun, Hammond and Sewell, 1981; Chapman and Mitchell, 1982a) have demonstrated that this resistance can also be passively transferred in sera from infected hosts. Most reports agree that the injection of metabolic products of immature flukes confers significant resistance to recipients, as does the implantation of these flukes into recipients (Lang, 1976; Lang and Hall, 1977; Rajasekariah, Mitchell, Chapman and Montague, 1979). Only a few workers (Ericksen and Flagstad, 1974; Anderson, Hughes and Harness, 1975) have reported that the implantation of the adult fluke will confer similar protection on recipients. It could be inferred that protective serum antibodies are directed against some components of the parasite's excretory/secretory products. If defined, such components will be useful targets for vaccination and/or diagnosis. This study was therefore designed to characterize *Fasciola hepatica* at the molecular level, with the aim of identifying parasite components that might be involved in host/parasite relationship in the rat, a suitable laboratory model for bovine fasciolosis.

CHAPTER TWO

LITERATURE REVIEW

2.1 THE PARASITE AND ITS LIFE CYCLE

The definitive hosts of parasitic trematodes of *Fasciola* spp. are mammals where they inhabit the liver bile ducts. Their eggs are passed into the bile and voided with the faeces of an infected host.

The life span of the miracidium is only about 24 hours, during which it must find and penetrate an intermediate host (Smyth, 1962). The most common intermediate host for *F. hepatica* is *Lymnea truncatula*, an amphibious snail while *L. natalensis* is the most common intermediate host for *F. gigantica*.

The life cycle of *Fasciola* spp. is fully described by Reinhard (1957) but, briefly, the miracidia undergo mitosis inside the snail and develop into a sporocyst. By repeated divisions, each germinal cell within the sporocyst becomes a germinal ball which then becomes a redia. Germinal cells within the rediae also develop into germinal balls, each of which gives rise to a second generation of rediae. The germinal cells of these second generation of rediae develop into cercariae. The tailed cercariae swim out of the snail and encyst on the surface of blades of grass and other objects, losing their tails in the process, to become metacercariae. The metacercaria is the infective form for susceptible mammalian hosts.

When metacercariae are ingested by a susceptible host, the outer cyst wall is digested off by the host's gastro-intestinal enzymes and the activity of the young fluke releases it through a pore in the inner wall. The newly excysted juvenile fluke penetrates the intestinal wall and passes via the peritoneal cavity (Schumacher, 1938) to the liver, into which it penetrates. These invading flukes migrate and grow for several weeks in the liver parenchyma, later entering into the bile ducts where they mature into egg-laying adults (Gorgi and Theodorides, 1980). The prepatent period for *F. hepatica* is about six weeks in the rat (Kendall, 1967), eight weeks in the sheep (Sinclair, 1962; Boray, 1969) and nine weeks in the cattle (Kendall, 1967). *F. gigantica* has a longer prepatent period of about 28 weeks in the cattle

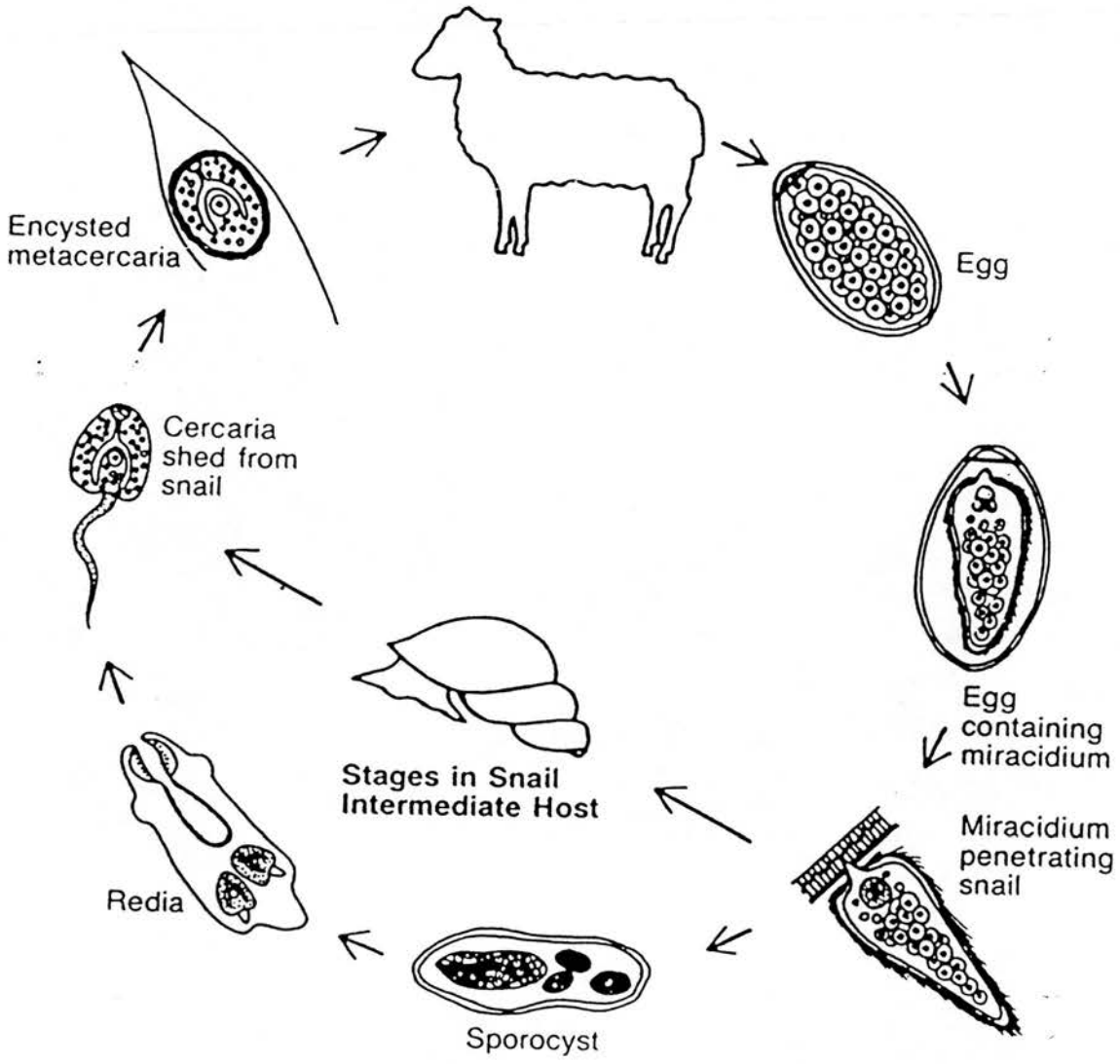


Figure 2.1.

Life cycle of *Fasciola* spp (after Urquhart, Armour, Duncan, Dunn and Jennings, 1988).

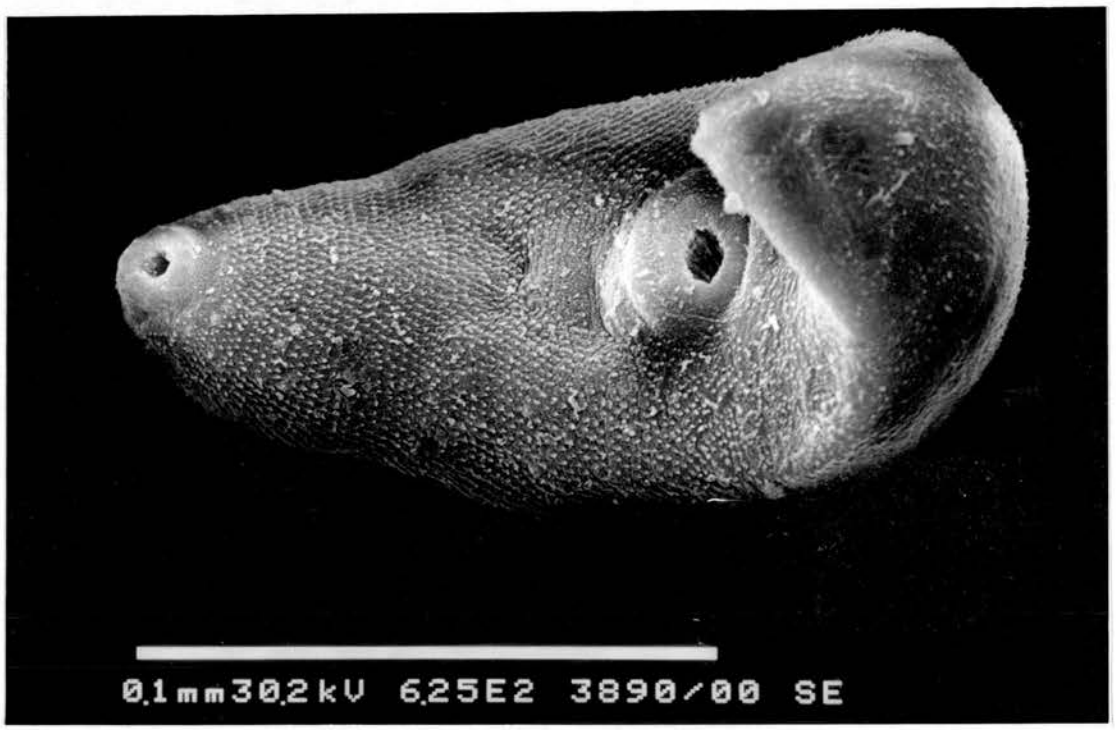


Figure 2.2.

Scanning electron microscopy of D14 F. hepatica (x 625).

(Ogunrinade, 1983) and 13 weeks in sheep and goats (Ogunrinade, 1984, Ajanusi, Ogunsusi, Njoku and Gyang, 1988).

2.2 RESISTANCE TO *FASCIOLA* INFECTION

Throughout this discussion the levels of protection are the results of comparison with animals which had not received the specified treatment but were otherwise alike in all aspects and had received the same dose of the same batch of metacercariae.

2.2.1 Resistance Following Natural Infection

2.2.1.1 Rats

The presence of immunity in rats with an existing infection derived from normal metacercariae was first demonstrated by Hayes *et al.* (1972), who observed that infection of rats with an existing infection of *F. hepatica* resulted in the recovery of 92.5% fewer flukes. This observation was confirmed by the same authors in 1973 when they found that 76% fewer flukes developed from a challenge infection in rats previously infected with one metacercaria of *F. hepatica* and that rats that had first been exposed to a primary infection of 10 metacercariae were able to completely resist a challenge infection. This resistance persisted even in chronic infections (Hayes *et al.*, 1974a), but the age of the primary infection at the time of challenge influenced the degree of resistance as rats challenged 28 or 48 weeks after a primary exposure yielded 66 or 50% less flukes respectively from challenge infection. Hayes and Mitrovic (1977) considered that the immunity is expressed in the rat within 24 hours of challenge. The number of flukes recovered 24 to 48 hours after challenge were 84 to 99% fewer. Doy, Hughes and Harness (1978) also reported that a 3-week-old primary infection of 30 metacercariae per rat initiated a high degree of resistance within 48 hours after challenge with 200 metacercariae. Anthelmintic removal of the immunizing infection before challenge did not interfere with the ability of the rats to develop resistance to the challenge infection. Goose

and MacGregor, (1973) demonstrated that a single sensitizing infection of rats with 30 metacercariae engendered resistance to a challenge infection given 63 or 98 days later as an oral metacercarial challenge or intraperitoneally transplanted adult flukes and that removal of the immunising infection by anthelmintic treatment 6 days before challenge did not interfere with the resistance. The effect of oral sensitization upon intraperitoneal challenge was also investigated by Hughes, Anderson and Harness (1976). They found that 71% of the implanted flukes were killed in rats which had been sensitized by oral infection with metacercariae 10 weeks before being challenged by intraperitoneal implantation of adult flukes. In contrast, in rats sensitized by subcutaneous implantation of adult flukes prior to challenge, only 23% of the challenge flukes were killed. Histological evidence suggested immunological killing of the challenge flukes. Therefore these authors concluded that oral infection of rats causes sensitization of the host against all stages of the parasite and that this is necessary for the development of protection against an adult fluke challenge. The work of later investigators (Rajasekariah and Howell, 1977, 1978; Haroun *et al.*, 1980b; Doy and Hughes, 1982) lends credence to this ability of rats to develop resistance after a primary infection.

Doy and Hughes (1982) observed that previously infected athymic nude Rnu/rnu rats and their heterozygous hairy (rnu/+) counterparts showed a high degree of resistance to oral challenge but that only the rnu/+ rats showed a high degree of resistance to an intraperitoneal challenge with newly excysted juveniles. In addition, they observed that serum from infected rnu/+ and PVG rats readily induced *in vitro* adherence of peritoneal cells of infected or naive PVG rats to newly excysted juveniles while serum from infected rnu/rnu rats failed to elicit adherence. They therefore postulated the existence of two distinct mechanisms of resistance, one a T-independent system effective at the gut wall and the second, a T-dependent system effective in the body cavity and the liver parenchyma.

2.2.1.2 Mice

Lang (1967) observed that mice immunized by two stimulating infections before challenge harboured significantly fewer flukes. There was earlier migration by the challenge flukes to the bile ducts. On the basis of the nature and timing of lymphocyte infiltration, and the histopathology of the liver, he suggested that delayed (cellular) hypersensitivity may play a prominent role in the earlier migration of the challenge flukes. Lang (1968) gave mice two immunizing infections of 2 metacercariae 60 days apart, followed by challenge 40 days later. At post mortem examination 25 and 40 days after challenge there was a significant decrease in worm burdens. As the author had been unable to demonstrate a significant decrease in the worm burdens in previously infected mice 20 days after challenge in a previous experiment, he concluded that the factors responsible for the immunity are manifested between 20 and 25 days. Lang's work was criticised by Dawes and Hughes (1970) on the grounds that he did not include the results obtained from all the mice which had received the two immunizing infections before challenge. However, Lang and Dronen (1972) again observed significant immunity in mice harbouring natural infection prior to challenge. Harness, Doy and Hughes (1977) did not find any significant decrease in the worm burdens of mice that had received a 3-week sensitizing oral infection of 2 metacercariae which was terminated by an anthelmintic prior to challenge. However, they did observe that significantly more flukes reached the liver 2 days after challenge in the sensitized mice.

2.2.1.3 Rabbits

Healy (1955) reported that in rabbits that had been infected with 50 metacercariae 60 days prior to an oral challenge there were no decrease in fluke burdens, although there were reduction in the size of challenge flukes recovered. However, Ross (1966) observed that in 4 rabbits that were each challenged with 50 metacercariae 24 weeks after a primary infection of 75 metacercariae, there was a

reduction in the number and size of challenge flukes recovered. However, there was much individual variation.

The effect of a primary infection on a subsequent challenge infection in rabbits was also investigated by Kendall, Hebert, Partiff and Pierce (1967). They observed that fewer flukes were recovered from a challenge infection of 20 metacercariae in rabbits with a 12-week-old primary infection of 10 metacercariae. However, these authors derived their conclusion from consideration of the number of flukes recovered when the primary and challenge infections were given separately.

Kendall and Sinclair (1971) confirmed that a primary infection did not engender resistance to a challenge infection in rabbits, but that termination of the primary infection with an anthelmintic 2 days before challenge resulted in a significant decrease in challenge fluke recovery. They attributed this decrease to the effect of the anthelmintic. Conversely, Bolbol, Hammond and Sewell (1978) found no reduction in the number of flukes recovered from rabbits challenged with 100 metacercariae 4 weeks after the removal of a primary infection of either 100 or 500 metacercariae. However, a significant decrease was obtained in rabbits that had two similar previous infections and treatments before challenge.

Fortmeyer (1973) found that rabbits that had each received an oral sensitizing dose of 50 metacercariae 28-263 days before an oral challenge of 50-150 metacercariae showed considerable reduction in challenge fluke burden. Fortmeyer (1974) again demonstrated that intraperitoneal infection of rabbits with 20 to 30 metacercariae 42 days before an oral challenge of 50 metacercariae did not result in immunity but that a marked immunity was produced when the interval between infection and challenge was increased to 94-155 days.

2.2.1.4 Sheep

Sinclair (1962) found that a group of sheep given four doses of metacercariae developed more severe anaemia, showed a lower rate of liveweight

gain but had similar fluke burden as a comparable group given the same number of metacercariae in a single dose. The findings of Boray (1967) also demonstrated that a 12-week-old primary infection of 200 metacercariae in sheep did not cause any decrease in fluke burdens from a challenge infection of 200 or 4000 metacercariae given 12 weeks later.

Sinclair (1970) studied the pathogenicity of *F. hepatica* after reinfection with 500 metacercariae in two groups of lambs, one of which had received daily injections of corticosteroid during the initial infection. Evidence of acquired resistance to reinfection was demonstrated in both groups by the recovery of fewer but larger flukes. However, there was considerable individual variation which precluded the demonstration of any statistical significance.

The role of the developmental stages in the resistance of sheep to reinfection was investigated by Sinclair (1971a). Comparing the development and pathogenicity of *F. hepatica* in previously uninfected sheep and in sheep in which a four-week-old infection of 300 metacercariae had been terminated by anthelmintic treatment 5 weeks prior to challenge, he observed a delayed onset of anaemia and later appearance of haemorrhagic lesions in livers of reinfected sheep. He concluded that the flukes remained longer in the livers of the reinfected sheep than in controls and that this retardation was a measure of resistance even though fluke recoveries were similar in both groups.

Sinclair (1973) confirmed these results in a study in which he again compared the development and pathogenicity of *F. hepatica* in three groups of sheep. Temporary retardation of development of flukes derived from the challenge infection was seen in the group that had been treated to eliminate a nine-week-old primary infection and in the group that had previously received five one-week long drug-abbreviated infections. However, the total recovery of flukes from both of these groups were similar to controls.

The results of Sinclair (1971a, 1973) were also supported by Rushton (1977) who did not observe any reduction in the worm burdens derived from challenge infections in sheep that already harboured an 11-week-old primary infection or in sheep in which a 10-week-old primary infection was removed before challenge. However, the reinfecting flukes had temporary retardation of growth.

Sinclair (1975) further studied the resistance to pathophysiological effects of a challenge infection in 3 groups of sheep, one previously unexposed, one exposed to a 15-week drug abbreviated infection of 1000 metacercariae, and the third exposed to five one-week drug abbreviated infections of 200 metacercariae. He found no evidence that previous infection conferred resistance to the pathophysiological effects of a challenge infection as both challenge groups showed earlier and greater peaks of eosinophils and a more gradual but persistent rise in glutamate dehydrogenase. In addition, there were no differences in fluke burdens of the three groups. Sinclair considered that this showed that the immune response in repeatedly infected sheep is ineffective in protecting them from challenge. Sandeman and Howell (1981) were also unable to demonstrate resistance to reinfection in sheep.

2.2.1.5 Cattle

The ability of cattle to acquire resistance to reinfection has been demonstrated by many workers (Ross, 1966; Boray, 1969; Doyle, 1971). However, Ross (1967) found that 3-month-old calves given an infection of 200 metacercariae three weeks before challenge with 300 metacercariae had no significant reduction in the total numbers of flukes recovered. However, a significant reduction in total fluke burden was obtained when the challenge infection was given 18 weeks after the primary infection. Elimination of a primary infection with anthelmintic does not interfere with the ability of cattle to acquire resistance to reinfection as Boray (1967) reported that eliminating a primary infection 3 weeks before challenge still resulted in a reduction of about 78% in fluke burden in previously infected steers. Previously infected steers also showed fewer clinical signs, lower faecal egg output

and a short patency in the challenge infections. Armour, Dargie, Doyle, Murray, Robinson and Rushton (1974) and Kendall, Sinclair, Everett and Partiff (1978) were also of the opinion that resistance in cattle persists after the elimination of a primary infection. Doyle (1973) found a relationship between the duration of the primary infection and the subsequent development of the acquired immunity. He observed a significant resistance in calves challenged after 12 but not in others challenged after 7 weeks of a primary infection of 750 metacercariae. He excluded the possibility of the resistance being due to physical factors such as the degree of fibrosis or chollangitis. Doy and Hughes (1984) also agreed that the duration of the primary infection influences the degree of acquired resistance but contrary to the opinion of Doyle (1973), these authors believed that physical barriers in the livers of infected cattle could not be ruled out as a mechanism of resistance to reinfection.

2.2.2 Stimulation of Resistance by Implanting Flukes

Protective immunity has generally been found to be best stimulated by living parasites and it is thought that the antigens responsible for this immunity are released by the parasites during their development in the host (Rickard and Bell, 1971a, b, c). Many attempts have been made to stimulate immunity to infection with various helminths by implanting the particular parasite in a site different from its normal predilection site in the host so that they can release antigenic metabolic products without causing significant pathological changes. Rickard and Bell (1971b, c) explained that when activated embryos of *Taenia taeniaformis* and *Taenia ovis* contained in membrane diffusion chambers were intraperitoneally implanted into rats and lambs respectively, the "diffusible functional antigens" thus produced stimulated a high degree of resistance to subsequent challenge.

Eriksen and Flagstad (1974) reported that there was a 50% reduction in the fluke burden in rats that received subcutaneous implantation of four adult flukes 3 weeks before an oral challenge. In addition, the percentage of liver necrosis was approximately 50% lower in the implanted rats. Similarly, Anderson, *et al.* (1975)

observed a 34% reduction in the challenge fluke burdens of rats that were sensitized by subcutaneous implantation of one adult fluke before oral challenge. Hughes, Anderson and Harness (1976) observed that sensitization of rats by subcutaneous implantation of one adult fluke 14 days prior to intraperitoneal challenge with one adult fluke resulted in the deaths of 23% of the challenge flukes while stimulation by oral infection resulted in the deaths of 71% of adult flukes intraperitoneally implanted as a challenge. Hughes, Harness and Doy (1981) reported that intraperitoneal sensitization of rats with 10 newly excysted juveniles, six or 8 14-day-old flukes 3 weeks before intraperitoneal challenge with adult flukes led to the death of almost all the challenge flukes. The study of Rajasekariah and Howell (1978) lends credence to the ability of young flukes to confer protection. These authors revealed that there was a significant protection in rats that had received subcutaneous implantation of 40 metacercariae or four 4-week-old *F. hepatica* two weeks before an oral challenge. However, they did not find any protection in rats sensitized by implantation with two adult flukes. In contrast, Haroun *et al.* (1980a) did find significant protection in rats that had received subcutaneous or intraperitoneal implantation of 2 adult flukes 2 weeks before an oral challenge. This protection was maintained even when the immunising flukes were removed by treatment before challenge. In addition, the immunised rats showed only little evidence of liver damage.

Lang (1974a) implanted 12, 14, 16, 18, 20 and 24 day-old *F. hepatica* intraperitoneally into groups of mice. The implanted mice were challenged orally when the transferred flukes had a total age of 40 days. A significant reduction in the fluke burdens was obtained in mice immunized with 12, 14, 16, and 18 day-old worms and in mice harbouring a natural infection before challenge. The author presumed that flukes older than 20 days do not arouse immunity or that the duration of the fluke's migration in the liver had an effect on it's ability to stimulate immunity. Lang and Dronen (1972) had earlier demonstrated protective immunity in

mice that had received an intraperitoneal implantation of 8 or 16 day-old flukes and challenged when the implanted flukes had a total age of 40 days.

Ross (1967) implanted 6-week-old flukes into the gluteal muscles of 6 rabbits, a lamb and a calf which had already received 5 subcutaneous injections of whole fluke homogenate. When these animals together with a group of animals that had received the whole fluke homogenate but not the implant and a control group which had received neither the homogenate nor the implant were concurrently challenged, there was no significant difference in the numbers of flukes recovered from the vaccinated and non-vaccinated groups although the vaccinated group (which had received the homogenate and the implant) had fewer flukes. A group of sheep in which 6-week-old flukes were similarly implanted in the intercostal muscles preceding challenge was also not protected.

2.2.3 Transfer of Immunity by Vaccination With Irradiated Metacercariae

Thorpe and Broome (1962) reported that in rats that were orally vaccinated with metacercariae irradiated at 2.5 kilo-roentgens 7 or 11 weeks before an oral challenge there was evidence of a significant immunity with a reduction of approximately 50% in worm burden. Although this result was criticised by Dawes and Hughes (1964) on the basis that the authors measured the degree of immunity by comparing their results to a "theoretical control group fluke burden", the findings nonetheless gained support from the work of Corba, Armour, Roberts and Urquhart (1971) and Armour and Dargie (1974). The former authors reported significant protection of 80% against oral infection in rats vaccinated with 3 weekly doses of metacercariae irradiated at 2.5 Kr but the latter authors obtained a much lower protection level (56%) in rats vaccinated with metacercariae that had been irradiated at 3 Kr. Hughes, Hanna and Symonds (1981) observed that sensitization of rats with a single oral dose of *F. hepatica* metacercariae irradiated at 3-8 Kr did not give significant protection against an intraperitoneal challenge with 3 adult flukes.

Hughes, Doy, Burden and Oldham (1982) demonstrated that rats sensitized for 3 weeks by a single oral dose of *F. hepatica* metacercariae irradiated at 3 Kr were significantly protected as demonstrated by a significant decrease in the fluke burdens recovered 48 hours after challenge. Sensitization using metacercariae irradiated at 4 Kr did not give significant protection against challenge.

Mice sensitized with *F. hepatica* metacercariae irradiated at 2-4 Kr (Hughes, 1962) or 3 Kr (Dawes, 1964) did not develop any significant resistance to a subsequent oral challenge. However, Harness, Hughes and Doy (1976) found that sensitization of mice for 3 weeks by one dose of *F. hepatica* metacercariae irradiated at 3.8 Kr led to significant (40%) decrease in the worm burden recovered 48 hours after challenge. In contrast, in a similar experiment, Harness *et al.* (1977) did not find a significant decrease in the fluke burdens of sensitized mice examined 12 or 14 days after challenge. They considered that the juvenile flukes resulting from the challenge infection migrated more quickly from the peritoneal cavity of previously sensitized mice and that this quicker migration may be due to the host's immune response in the peritoneal cavity or to "substances" leaking from the liver tissue already damaged by the sensitizing infection.

Sheep vaccinated with three doses *F. hepatica* metacercariae irradiated at 20 Kr were not significantly protected from a challenge infection (Dargie, Armour, Rushton and Murray, 1974). A similar observation was made by Campbell, Gregg, Kelly, Dineen (1978). However, Agadir, Haroun and Gameel (1986) reported significant protection against challenge in sheep sensitized 8 weeks before challenge with metacercariae of *F. gigantica* that had been irradiated at 3 Kr.

Boray (1967) reported that cattle immunized with 3 doses of *F. hepatica* metacercariae irradiated at 20 Kr 3 weeks before an oral challenge of 5000 metacercariae were not significantly protected although they showed fewer pathological changes in the liver. However, Armour *et al.* (1974) found a reduction

of 30% in the fluke burdens in a group of cattle challenged 4 weeks after vaccination with two doses of metacercariae of *F. hepatica* irradiated at 3.5 Kr. A reduction of 70% was found in the group challenged 8 weeks after immunization. Nansen (1975) reported that in calves that had been vaccinated with two doses of 1500 *F. hepatica* metacercariae irradiated at 3 Kr prior to their exposure to natural infection, there was a decrease of 71% in the fluke burden. Significant levels of protection (65-98%) have also been reported in calves that had received one or two doses of *F. gigantica* metacercariae irradiated at 3 Kr (Bitakaramire, 1973) or 2 Kr (Younis, Yagi, Haroun, Gameel and Taylor, 1986) 8 to 14 weeks before challenge. In calves challenged 4 weeks after sensitization only 18% protection was seen (Younis *et al.*, 1986).

2.2.4 Stimulation of Immunity by Somatic Fluke Extracts

The use of fluke extracts or fractions of such preparation for vaccination against fasciolosis have yielded conflicting results in different hosts. In most cases the results of such vaccination mirrors the natural ability of the host to develop resistance.

Hughes *et al.* (1981) did not find any protection in rats that had received two intramuscular injections of 0.2 ml of adult fluke somatic antigen three weeks before receiving an intraperitoneal challenge with 2 adult flukes. Similarly, Burden, Harness and Hammet (1982) were unable to demonstrate resistance in rats that had received a subcutaneous injection of 500 μg fluke protein from 14, 16 or 17 days old flukes. Although Oldham and Hughes (1982) did not find resistance in rats sensitized with adult fluke extract in Freund's adjuvant by the subcutaneous and intramuscular routes, rats sensitized with the antigen by the intraperitoneal route had significantly lower fluke burdens whether the extract was presented in Freund's incomplete adjuvant alone or in Freund's incomplete adjuvant with *Bordetella pertusis* (Oldham, 1983). However, Yoshihara, Nakagawa and Suda (1985) reported a 74% reduction in the number of flukes recovered from the liver of rats that were

given two doses (intramuscular and subcutaneous) of a metacercarial extract containing 400 μg of protein two weeks before an oral challenge. Rats immunized with the same doses of adult extract had a 40% reduction in fluke burdens.

Lang (1976) was unable to confer resistance to infection in mice following intraperitoneal injection of a homogenate of 16-day-old flukes. However, Lang and Hall (1977) did appear to stimulate resistance in mice that had received one or two intraperitoneal injections containing 500 μg of a somatic preparation obtained by sonic disruption of freshly collected 16-day-old flukes. These vaccinations resulted in 86 and 82% reduction in worm burdens respectively. In contrast to these results, Chapman and Mitchell (1982a) found no significant resistance in mice that had been sensitized with 3 subcutaneous doses of sonicated newly excysted juvenile or 2 doses of sonicated 16-day-old flukes before challenge although the vaccination did lead to lower mortality rates. Hillyer (1985) vaccinated mice with the *Fasciola/Schistosoma* cross reactive antigen, so called Fhsm(111) isolated from adult *F. hepatica*. The immunization conferred 69-78% protection. Immunization using crude adult *F. hepatica* extract conferred a protection of 15-31% on recipient mice.

Kerr and Petkovich (1935) immunized 7 rabbits with nine 1 ml doses of intraperitoneal injections of a 1% suspension of adult flukes five weeks before an oral challenge with 13 metacercariae. The mean number of flukes recovered from the immunized group was 2.1 as compared to a mean of 8.3 recovered from the non-immunized controls. However, Urquhart, Mulligan and Jennings (1954) questioned the significance of these results on the basis of the small number of animals used. These latter authors were unable to find resistance in rabbits immunized with three intramuscular doses of alum-precipitated protein extract prepared from a crude adult fluke homogenate, apart from evidence of retarded development of the flukes recovered from the immunized rabbits. As in the results of Urquhart *et al.* (1954), Healy (1955) found no evidence of resistance against reinfection in rabbits

vaccinated with whole fluke extracts containing large amounts of polysaccharides but no proteins or lipids. Ross (1967) found no resistance in rabbits sensitized by two subcutaneous injections of homogenate of 6-week-old flukes three weeks before challenge. However, the findings of Sinclair and Joyner (1974) were similar to those of Kerr and Petkovich (1935). They reported that in rabbits sensitized by both an oral primary infection and intravenous injection of an adult fluke extract there was 50 to 75% reduction in the fluke burdens which developed from a challenge infection. This significant protection was not achieved when rabbits were sensitized only with the fluke extract.

Ershov (1959) reported that only 10-25% of sheep vaccinated with a fluke-antigen complex containing 60% polysaccharide and 30 to 40% albumen developed a solid resistance, while the rest only showed reduced susceptibility. However, this immunity was short-lived as no resistance was found in any vaccinated sheep infected 45 days after vaccination. Absence of resistance was also reported by Ross (1967) in a lamb and a calf vaccinated with 5 subcutaneous injections of whole fluke homogenate, but Hall and Lang (1978) reported protection of 90.6, 98 and 99% in calves sensitized by a single subcutaneous dose of sonicated 16-day-old flukes for 38, 100 and 200 days respectively. Hillyer, Haroun, Hernandez and Soler De Galanes (1987) also achieved a 55% reduction in fluke burdens in cattle that were each immunized with 1 mg of a *Fasciola/Schistosoma* cross reactive antigen 7 weeks prior to an oral challenge.

2.2.5 Stimulation of Resistance by Metabolic Products

The metabolic products of helminths cultured or maintained *in vitro* have often been used in attempts to stimulate immunity against these parasites (Soulsby, 1957, Urquhart, Jarrett and Mulligan, 1962).

Rats have been successfully vaccinated using the immune complex formed by culturing metacercariae in immune or hyperimmune homologous serum (Howell, 1979; Howell and Sandeman, 1979) but not by culture products obtained from 48-

hour or 14-day *in vitro* cultivation of newly excysted juvenile (Davies, Rickard, and Hughes, 1979). Rajasekariah *et al.* (1979) reported that a degree of resistance comparable to that seen following a low-dose oral infection could be induced in rats by subcutaneous injection of ES products (1.6 mg) harvested from 4-week-old but not from 8-week-old *F. hepatica*. Burden and Hammet (1980) did not observe any resistance in rats sensitized on days 0 and 12 by subcutaneous injection of unembryonated or macerated *F. hepatica* eggs or ES products from adult flukes 2 days before an oral challenge. However, the flukes recovered from the rats vaccinated with ES products were smaller than those from the controls. Analogous results were obtained by Burden *et al.* (1982). These authors injected 500 μg of ES proteins from 16-day-old flukes subcutaneously and intraperitoneally into rats 3 weeks before an oral challenge of 20 metacercariae and were unable to demonstrate any significant protection. The authors also revealed that subcutaneous injection of rats on days 0 and 14 with concentrated incubation medium (containing 1.6 mg protein) from 10 or 13-day-old flukes caused slight but insignificant reduction in fluke burdens resulting from an oral challenge given two weeks after the last immunising dose.

Lang (1976) used metabolic products from 16-day-old *F. hepatica* maintained in medium 199 for various periods of time as a vaccine against *F. hepatica* in mice. A single intraperitoneal injection of medium in which 40 16-day-old flukes had been maintained (at 150 μl of medium per fluke) for 4 hours and which contained 160 μg of protein, failed to stimulate resistance in recipient mice, although host mortality was significantly lowered. On the other hand, four sequential vaccinations (total dose of 1920 μg per mouse) using worm incubate produced over a 24-hour period protected 75% of the mice from challenge and reduced the worm burden by 83.3%. Lang and Hall (1977) vaccinated groups of mice with incubation media produced over various periods of time and found that the highest resistance was shown by the group vaccinated with medium collected

between 12 and 24 hours after incubation commenced. As the injection of medium collected at 24 hours produced only marginally significant immunity, the authors suggested that qualitatively different antigens were produced between 12 and 24 hours. Contrary to the results of Lang (1976) and Lang and Hall (1977), Lehner and Sewell (1979) found that vaccination of mice with incubation medium from 16-day-old flukes (375 µl of medium/fluke) did not result in protection. Rajasekariah *et al* . (1979) were also unable to demonstrate protection in mice that had been sensitized intraperitoneally or intramuscularly with ES products from 4-week-old *F. hepatica* 14 days before challenge.

Healy (1955) attempted to vaccinate rabbits against fasciolosis using pooled regurgitated caecal contents of adult flukes. No resistance was found in the vaccinated rabbits 60 days after challenge. However, some of the flukes recovered from the vaccinated rabbits showed retarded development and produced abnormal eggs. Similarly, Lehner and Sewell (1979) did not observe resistance in rabbits sensitized four times with varying doses of metabolic products from immature and mature adult *F. hepatica* maintained in a recirculating system for 7 days. They did observe however that flukes recovered from the vaccinated rabbits were slightly smaller in size.

2.2.6 Passive Transfer of Immunity by Serum Injection

2.2.6.1 Rats

Corba *et al.* (1971) reported that intraperitoneal injection of naive rats with 4 ml of homologous serum obtained from rats 8-10 weeks after infection of 10 *F. hepatica* metacercariae did not protect against challenge. However, Armour and Dargie (1974) later demonstrated the ability of homologous and heterologous serum to confer protection on recipient rats. They found that naive rats intraperitoneally injected on the day of challenge and again two days later with 10 ml of serum obtained from infected rat, had an overall reduction of 71% in their fluke burdens while those injected with similar volumes of serum from sheep or cattle with 12-14-

week-old primary infection of 1000 metacercariae had reductions of 79 and 66% respectively. The authors demonstrated that the volume of serum influenced the degree of protection. Thus, the mean number of flukes recovered from rats given 0, 5, 10 or 20 ml of serum were 3.6, 2.0, 1.4 and 0.6 respectively. Hayes *et al.* (1974c) did not obtain a similar relationship in their studies. They found all groups of rats that received 1, 2.5 or 5 ml of homologous antiserum at the time they were challenged had significant reductions in their fluke burdens. This protective effect was removed by heat treatment and adsorption with live flukes. Howell, Sandeman and Rajasekariah (1977) demonstrated that a significant protection was conferred on naive recipients by serum from donor rats that had received primary and challenge infections of *F. hepatica* metacercariae. In addition, the authors observed that exposure of newly excysted juveniles to immune rat serum resulted in complete loss of viability. Rajasekariah and Howell (1979) observed that, while serum from rats that had experienced 10-15 weeks of infection with 5 metacercariae did not protect recipient rats from challenge, serum from similar rats with a secondary infection of 30 metacercariae conferred significant protection to the recipients. The ability of serum from infected rats to confer protection on recipients is also supported by the results of Haroun *et al.* (1981) and Pfister, Turner and Wedrychowicz (1984/85).

There is a relationship between the ability of immune homologous serum to confer protection and the time at which the serum is administered to recipient rats. Hayes *et al.* (1974c) observed that antiserum given 2, 4, 6 or 8 days after challenge did not result in significant resistance, although there appeared to be a slight protective effect following transfer 2 or 4 days after infection. Similarly, Chapman and Mitchell (1982a) observed that no protection was conferred when the serum was given 14 days after infection. Antiserum collected from rats with chronic infections had no protective effects on recipients (Howell *et al.*, 1977).

Chapman and Mitchell (1982a) established that heated immune rat serum was as effective as unheated serum. The authors deduced that the heat-labile serum

components such as IgE and complement were not responsible for serum-mediated transfer of protection in rats. This observation on the effect of heat on immune serum was a direct contrast to that made earlier by Hayes *et al.* (1974c).

Immune ovine serum has been shown to confer significant protection on recipient rats. Armour and Dargie (1974) obtained a significant reduction (66%) in the fluke burdens of rats that had received antiserum obtained from sheep infected 12 to 14 weeks previously with 350 *F. hepatica* metacercariae. The ability of ovine antiserum to confer protection in recipient rats was also demonstrated by Mitchell, Armour, Ross and Halliday (1981) and Sandeman and Howell (1981). The latter authors reported that significantly fewer flukes were recovered from rats into which juvenile flukes were injected after they had been cultured in immune serum obtained from sheep 10 weeks after a primary infection. Serum obtained 12 weeks after infection had no effect on juvenile flukes.

Rats have also been protected by serum obtained from infected rabbits (Haroun *et al.*, 1981) and cattle (Corba *et al.*, 1971; Haroun *et al.*, 1981) but not mice (Chapman and Mitchell, 1982a).

Armour and Dargie (1973), demonstrated that IgG fraction was involved in the transfer of this passive immunity. Rats that were injected with this fraction had significant (55%) protection. Such protection was not found when IgM was transferred. Dargie, Armour and Urquhart (1973) also described transfer experiments using fractions of immune serum from rats and cattle and indicated that IgG was a pre-requisite for successful protection.

2.2.6.2 Rabbit

Baalawy (1975) transferred 1 to 10 ml of concentrated homologous immune serum to rabbits at the time of challenge and 2 days later. The immunization conferred a protection of 25 to 40% against an oral challenge with *F. gigantica*. The protection conferred by the serum was directly related to the infecting dose of metacercariae given to the donor rabbits. Thus, the highest protection (40%) was

obtained in rabbits which received 10 ml of serum obtained from rabbits infected with 30 metacercariae. The same dose of concentrated immune serum from goats infected with 250 metacercariae also conferred the highest (78%) resistance. This author concluded that for immune serum to be effective, it must be given in high concentration, in a sufficiently high dose and it must be collected while the young flukes are still in their migratory phase. This author did not indicate how "concentrated" the serum should be to be effective. Haroun *et al.* (1981) observed significant protection in rabbits that had received heterologous rat or bovine antiserum but not homologous antiserum at the time of challenge.

2.2.6.3 Mice

Lang (1974b) found that when 12, 16, 18, 20 or 24-day-old flukes were incubated in homologous immune serum and intraperitoneally transferred to recipients mice, there was a significant reduction in the worm burden which resulted from a subsequent oral challenge when compared with that from mice implanted with flukes incubated in heat-inactivated immune serum, normal serum, medium 199 or buffered saline. In contrast to the result of Chapman and Mitchell (1982a) this author observed that effect of immune serum was significantly decreased by heat inactivation at 56°C for 30 minutes, and suggested that complement was necessary for the antigen-antibody reaction involved in this type of protection. The significant reduction in the length of flukes recovered from mice implanted with flukes incubated in inactivated immune serum led the author to suggest that the effect is not only due to IgG, as had been suggested by Dargie *et al.* (1973) but also to IgM. The IgM, they suggested, may combine with a cell membrane antigen, activating complement to produce immune lysis of cells and death of flukes.

Lang (1976) incubated 16-day-old *F. hepatica* in immune serum from mice which had been injected with ES products from 16-day-old flukes or in immune serum from mice with 25-day-old infections prior to intraperitoneal transfer to recipient mice. At necropsy, it was found that in both cases the immune serum had a

protective effect, shown by a significant decrease in both fluke recovery and host mortality. Serum from mice which had been injected with a somatic antigenic preparation or from those with 100-day old infections had no such effects. Hence, the author suggested that those ES antigen(s) which stimulate the production of antibodies harmful to the flukes are produced only by immature flukes. Following the observation that incubation of young flukes in antiserum from mice with a mature infection resulted in earlier migration after transfer, the author suggested that mature flukes also produce the antigen concerned but that their location in the bile ducts does not allow these antigens to have sufficient contact with the host to maintain immunogenic levels. However, Chapman and Mitchell (1982a) indicated that serum from infected mice did not protect homologous recipients against an oral challenge.

2.2.6.4 Sheep and cattle

Although sheep are naturally unable to develop resistance to reinfection, serum from infected sheep has been shown to confer protection in some recipient hosts. Serum transfer experiments using homologous immune ovine serum are rare. Armour and Dargie (1973) reported that sheep could be protected against *F. hepatica* if large volumes of immune serum are used. They found that 400 ml of immune serum injected intraperitoneally resulted in 80% protection against a challenge of 750 metacerciae.

In an experiment involving the transfer of 2400 ml of hyperimmune serum from a donor calf with a mature *F hepatica* infection into the peritoneal cavity of recipient calves simultaneously challenged with 1000 metacerciae, Corba *et al.* (1971) did not find significant protection. However, Armour and Dargie (1973) reported that cattle could be protected if larger volumes of serum were given. They reported that immune serum administered intraperitoneally to cattle at 3.6L/250 Kg body weight resulted in 99% protection against an oral challenge of 1000 metacercariae.

Transfer of immunity by serum is traditionally taken as evidence of antibody involvement (Jones, Edwards and Ogilvie, 1970). The mechanism for this protective effect are not entirely known. Doy *et al.* (1978) and Doy and Hughes (1982) observed immune adherence of peritoneal cells onto newly excysted *F. hepatica in vitro* in the presence of immune serum but this did not affect their viability when subsequently transferred into naive rats. By contrast, Davies and Goose (1981) observed that newly excysted juvenile *F. hepatica* injected into previously sensitized rats were rapidly coated with peritoneal cells, which were releasing peroxidase and other lysosomal enzymes onto the worms, which were eventually killed. Furthermore, Eckblad, Woodard and Ogilvie (1981) detected, *in vitro*, structural damage to the tegument of juvenile (16-day-old) flukes caused by serum antibodies from a fluke-infected calf. Moloney and Denham (1979) observed that new born larvae of *T. spiralis* exposed to immune serum before infection of mice had significant decrease in infectivity, while new born larvae exposed to adsorbed immune serum were unaffected. They suggested that exposure to immune serum *in vitro* makes the larvae chemotactic to some type of "killer cell" and that this cell prevents them from infecting the muscle.

2.2.7 Transfer of Resistance by Sensitized Cells

Lang, Larsh, Weatherly and Goulson (1967) demonstrated protective immunity to *F. hepatica* in mice that had received intraperitoneal injections containing peritoneal exudate cells obtained from mice harbouring 35-week-old infections. Significantly fewer flukes developed following a challenge infection of the vaccinated mice, and they had a more rapid cellular (polymorphonuclear) response. The authors suggested that a delayed sensitivity reaction had taken place, resulting in allergic inflammation, rapid migration of the young flukes and death of some of the flukes.

Intraperitoneal transfer of lymphoid cells from infected rats to syngeneic recipients has been shown by several authors to give significant protection against

an oral challenge infection (Corba, *et al.*, 1971; Armour and Dargie, 1974; Rajasekariah and Howell, 1979). However, Pfister *et al.* 1984/85) did not observe any protection in rats given lymphoid cells obtained from rats infected with *F. hepatica* for six weeks. Rajasekariah and Howell (1979) found that lymphoid cells obtained from splenic, mesenteric and hepatic lymph nodes from immune and hyperimmune donor rats conferred protection on recipients examined at 4 weeks but not at 8 weeks after challenge although they did not mention the number of cells transferred. The authors agreed that this was a conflicting result and suggested it might have arisen due to small numbers of experimental animals. Mitchell *et al.* (1981) obtained a 55.6% reduction in the fluke burdens in rats that had been intraperitoneally injected with leucocyte lysates obtained from infected rats although they did not mention the number of cells in the lysate.

Baalawy (1975) obtained a 53.5% protection against oral challenge in rabbits immunized with 2 x 10^9 lymphocytes from infected homologous donors.

Homogenates of lymph nodes and spleen from donor sheep infected with *F. hepatica* for 8 weeks did not reduce the number of flukes which developed after challenge although some retardation in fluke development was observed in the recipient sheep (Sinclair, 1971b).

Corba *et al.* (1971) obtained significant protection in a calf that had received lymphoid cells obtained from its infected monozygous twin.

The basis of transfer of resistance by sensitized cells is not well understood. Eosinophils (Doy *et al.*, 1978; Milbourne and Howell, 1990), neutrophils and macrophages (Bennett, Hughes and Harness, 1980) have all been implicated as effector cells in resistance to *F. hepatica* in sensitized rats.

Eosinophilia, is a major hallmark of many helminth infections (Butterworth, 1984), especially of those with tissue-invasive stages (Befus and Bienenstock, 1982). Milborne and Howell (1990) demonstrated that rats and mice infected with *F. hepatica* or injected with ES products of adult fluke showed a rapid increase in

peripheral eosinophil as well as increase in the percentage of bone marrow proeosinophil cells. In rats infested with intestinal worms, localized accumulation of eosinophils in the lamina propria and submucosa may arise from chemotactic factors released following mast cell/IgE-worm antigen interaction, localized T-cell-mediated eosinophilopoesis and chemoattractants released from the worms themselves (Moqbel and MacDonald, 1990).

Lang (1967; 1968) suggested that sensitized lymphocytes may be attracted to sites of antigen deposition, where they initiate a delayed-type hypersensitivity reaction which renders the environment unsuitable for the young flukes. During a primary infection in rats, Keegan and Trudgett, (1992) observed pronounced eosinophilia in peripheral blood and massive eosinophil infiltration and degranulation in sections of the infected liver. In addition, peroxidase was present at the site occupied by the parasite and also on the fluke's tegument. Peroxidase catalyses oxidation of many substances by hydrogen peroxide and oxidation, coupled with the participation of halides, markedly potentiates the killing of metazoan parasites by phagocytes (Letonja and Hammerberg, 1987). The study by Keegan and Trudgett (1992) also revealed acid phosphatase staining in liver from rats that had been infected with *F. hepatica* for 3 weeks, which the authors said was an indication of the presence of large numbers of activated phagocytes.

Chapman and Mitchell (1982b) showed that immature *F. hepatica* releases a papain or cathepsin B-like thiol protease which cleaves mouse, rat, rabbit or sheep immunoglobulins *in vitro*. They suggested that this cleavage of immunoglobulin could render one of the major host defence systems ineffective in the vicinity of the parasite. Such an evasion mechanism has been postulated for the non-parasitic *Taenia pysiformis* (Eisen and Tallan, 1977) and *S. mansoni* shistosomula (Auriault, Ouiassi, Torpier, Eisen and Capron, 1980). Auriault *et al.* (1980) also indicated that in addition to rendering IgG non-functional, the peptides released by IgG cleavage inactivate rat macrophages, thus paralysing another host defence system.

2.3 ANTIGENS OF HELMINTH PARASITES

The variety of different antigens to which the host is exposed during helminth infections has been classified into four major levels. These are the parasite stage, the antigenic compartment within the stage, the antigenic components within the compartment and antigenic epitopes of each particular antigen. The antigens in any given stage of a parasite can be classified in three compartments, surface, secretory and somatic (Parkhouse and Harrison, 1989).

The antigens in the first two compartments play important role in the host-parasite relationship since they form the interphase between the living parasite and the host's immune response. In particular, antigens in the surface compartment are the direct target of the host immune responses and components within this compartment could be important in parasite protection. Thus, purified surface antigens of the infective larvae of the nematode *Trichinella spiralis* have been shown to be protective in mice (Silberstein and Despommier, 1984,1985; Gamble, 1985.). A monoclonal antibody produced against newborn larvae of this parasite has also been demonstrated to confer passive protection against infection (Ortega-Pierres, MacKenzie and Parkhouse, 1984). Monoclonal antibodies directed against determinants on the surface of hatched-activated *Taenia saginata* onchospheres have been used successfully in passive protection of cattle against experimental oral infection (Harrison and Parkhouse, 1986).

The term excretory/secretory product is used to describe material collected from helminths *in vitro* and shown to be produced or assumed to be produced *in vivo* and an extensive review on the subject was given by Lightlowers and Rickard (1988). There are various sources of excretory/secretory products. These include excretory glands and oesophageal glands of several nematode species (Edwards, Burt and Ogilvie, 1971; Ogilvie, Rothwell, Bremner, Schnitzerling, Nolan and Keith, 1973), stichocyte cells surrounding the oesophagus in members of the

superfamily Trichuroidea (Despommier and Muller, 1976; Jenkins and Wakelin, 1977), apical and lateral glands in schistosome miracidia (Hang, Warren and Boros, 1974), scolex of taeniid parasites (Heath, Lawrence, Glennie and Twaalhoven, 1985; Jenkins and Rickard, 1986a,b). In addition, molecules derived from the tegument or cuticle may make a substantial contribution to the contents of ES products of parasites maintained *in vitro* and are probably shed *in vivo* (Kusel, MacKenzie and McLaren, 1975; Wilson and Barnes, 1977).

A variety of functions, some of which are apparently protective to the parasite, have been associated with ES products of helminths. Species of some nematodes are known to secrete enzymes with acetylcholinesterase activity *in vitro* (Edwards *et al.*, 1971; Ogilvie *et al.*, 1973; Bremner, Ogilvie, Keith and Berrie, 1973). The secreted enzymes suppress local peristalsis, thereby allowing the worms to remain at their predilection sites. In addition the enzyme may also interfere with or delay worm expulsion by controlling acetylcholine-induced secretion of mucus goblet cells in the gut. ES products may also have a suppressive effect on lymphocyte function. For instance, *Taenia taeniaformis* ES products have inhibitory effects on lymphocyte function *in vitro* through induction of suppressor cell populations (Burger, Rikihisa and Lin, 1986). Similarly, the caecal content of adult *F. hepatica* have been shown to be toxic for rat splenic lymphocytes and to inhibit the binding of rat peritoneal cells to the parasite (Goose, 1978). The transformation of *Schistosoma mansoni* cercariae into schistosomula is associated with the release of certain factors which have been shown to exert inhibitory effects on peripheral blood mononuclear cell proliferative responses to phytomitogens (Vierra, Gazzinelli, Kusel, De Souza and Colley, 1986).

The gut associated ES products of adult schistosomes are significant components of the circulating immune complexes which are known to cause blockading of antibody-dependent cytotoxicity of eosinophils for *S. mansoni* schistosomula. The ES products of *T. spiralis* has been shown to contain the enzyme

superoxide dismutase (Rhoads, 1983) and marked differences in variation in susceptibility of different life-cycle stages of this parasite to *in vitro* killing by granulocytes and oxidant-mediated damage are reflected in their superoxide dimutase content (Kazura and Meshnick, 1984). Co-incubation of the highly susceptible newborn larvae with oxidant-resistant adult worms resulted in partial protection of the susceptible newborn larvae.

Ironically, parasite products may sometime have detrimental effects against the parasite. When surface molecules are released into ES products, it may stimulate immune responses against the parasite surface. Such potential is suggested by the demonstration that a monoclonal antibody raised against a 64 kDa antigen present on the surface of, and released *in vitro* by, newborn larvae of *Trichinella spiralis*, mediates eosinophil killing of larvae *in vitro* and reduces the infectivity of antibody-treated larvae *in vivo* (Ortega-Pierres, *et al.*, 1984).

2.4 "ANTIGENS" OF *FASCIOLA* SPP

An understanding of the antigenic composition of the various stages in the life cycle of *F. hepatica* is of importance to understudy host/parasite interactions and development of diagnostic assays. A defined sequence in the development of these antigens seems to occur as the fluke develops. (Sewell, 1964; Sinclair and Kendall, 1969; Gundlach, 1971).

On its way to the bile ducts, the migrating fluke undergoes both morphological and physiological changes to its outer tegument or glycocalyx, which comprises an anucleate surface syncitium bound apically and basally by tubules to the underlying perikarya or tegumental cells (Threadgold, 1967; 1976). The newly excysted juvenile has only one type of tegumental cell (Bennett and Threadgold, 1973) referred to as the Type 0 cell. These cells are filled with a single type of secretory granule, a very dense and thick biconcave disc, 0.22 μm in diameter which is unlike either of the secretory bodies of the adult cell types and is therefore

designated as T0 granule. The cell also contain occasional masses of crystalline spine-like material. Between 5 days and 3 weeks the synthesis of T0 granules in the Type 0 tegumental cells gradually declines (Bennett and Threadgold, 1975). The tegument of the adult fluke is known to be composed of two types of cells, Type 1 and Type 2, both of which lay below the peripheral musculature and are connected to the surface syncitium. The Type 1 cell metamorphose from the T0 cell after arrival in the liver while the Type 2 cell metamorphose from the embryonic cells in the parenchyma (Bennett and Threadgold, 1975). The T1 bodies produced by the T1 cell are spheroidal and electron dense 0.12 μm in diameter, while the T2 bodies are flattened biconcave disks, 0.20 μm in diameter, with electron lucid contents. The T1 bodies are thought to serve to replace the surface glycocalyx of *F. hepatica* as it becomes complexed with host antibodies during the parenchymal stages of development (Hanna, 1980a). The synthesis and transportation of T1 bodies has been investigated by Hanna (1980b). When slices of adult flukes were incubated in [^{3}H]leucine the T1 tegumental cells showed labelling over the cytoplasm and after 1h incubation labelled T1 bodies were seen free in the cytoplasm of the T1 cells and in the connecting tubules leading to the surface syncitium. Carbohydrate moities were added to the proteins in the golgi fields to form the future secretory product (Hanna, 1976) and the resulting glycoprotein is concentrated and packaged into spheroidal electron dense bodies known as T1 bodies (Threadgold, 1976). The T2 cell synthesize the T2 bodies, which are flattened biconcave disks, 0, 20 μm in diameter with electron-lucid contents which are also glycoproteins.

2.4.1 "Antigens" of Newly Excysted Juvenile Fluke (D0)

DO flukes express stage-specific antigens as well as some which are shared with later developmental stages (Reddington, Wes Leid and Wescott, 1984). Davies *et al.*, (1979) revealed that infected rats synthesized antibodies against an antigen derived from D0 *F. hepatica* maintained *in vitro*. These *in vitro* culture antigens were thought to be at least partially stage-specific because, under the cultivation

conditions used, the flukes did not switch over to the production of adult tegumental and caecal granules. This line of thought was confirmed by *in vivo* studies (Hanna, 1979; 1980a) involving monitoring the antibody developing on the surface of developing flukes. Antisera from sheep and rats that had been infected for 6-7 weeks with *F. hepatica* reacted strongly with the tegument of young flukes but gave progressively weaker reactions with older flukes, while antisera from animals infected for longer than 6 weeks gave strong reactions with adult antigens and weaker reactions with antigens from young flukes. Yoshihara, Taira and Suzuki (1981) used immunodiffusion to study the reaction between metacercarial extracts and antisera from rabbits infected with *F. hepatica*. They reported two precipitins, one of which was also detected in extracts of immature and adult flukes. Using this same technique, Sandeman and Howell (1981) found that at least two juvenile "antigens" were present in the precipitates formed around juvenile flukes cultured in immune rat serum. Bennett, Joshua and Hughes (1982) immunized rabbits with soluble metacercarial extracts and adsorbed the resultant antisera by adult fluke extracts. By an immunofluorescent technique, they showed the presence of at least two unique, juvenile-specific antigens in the adsorbed antisera. One of these antigens was present up to day 4 after infection while the other persisted until 7 days after infection.

Bennett (1978), however, had been unable to detect any immunofluorescent labelling on the tegument of D0 flukes using antiserum raised in rabbits against a soluble extract of adult fluke even though reactions were detected on the surface of other juvenile stages.

Using ^{125}-I lactoperoxidase surface labelling, Reddington and Leid (unpublished result cited by Reddington, Leid and Wescott, 1984) showed that sera from infected cattle and goats recognised a minimum of 13 surface antigens in D0 flukes although the values of these antigens were not stated. Lammas and Duffus (1985) mentioned that iodogen labelling of D0 flukes revealed components with

molecular weights of 10.5, 13, 13.5, 26, 30, 45.5 and 78 kDa as the major components. Dalton and Joyce (1987) reported the identification, by surface (^{125}I and galactose oxidase/tritiated sodium borohydride) radio-labelling, that glycoproteins with weights of about 75, 90, 180 and 200 kDa were specific to D0 flukes (Table 2.1).

2.4.2 "Antigens" of Immature Stages

Thorpe (1965) was the first to show that sections of immature and mature flukes treated with fluoresceinated globulins from infected rats showed strong immunofluorescence in the caecal lining, excretory ducts and the cuticle. Moore and Halton (1976) found that carboxylesterase, acetylcholinesterase, and alkaline phosphatase were similar in both immature and mature flukes. Antiserum raised in rabbits against a soluble extract of adult fluke reacted with all but the earliest forms of the immature fluke. The reaction was strongest with migrating flukes while the tegument of newly excysted juveniles or of stages recovered 1 and 2 days after infection from murine livers were not labelled (Bennett , 1978). Sandeman and Howell (1981) identified two antigens by gel diffusion of an extract prepared from immature fluke reacted against serum from infected sheep.

Of the seven surface-labelled components that were identified on newly excysted juvenile (Lammas and Duffus, 1985) that of 13 kDa was not present in 7-day-old flukes and in 14-day-old flukes there was additional loss of the 26, 30 and 78 kDa components and the appearance of a 32 kDa component (Table 2.1)

Dalton and Joyce (1987) reported that surface-radio-labelled glycoproteins of 180 and 200 kDa present in D0 flukes were not detected in D21 flukes but that the latter contained four specific components of 70, 85, 94 and 110 kDa (Table 2.1).

Dalton, Tom and Strand (1985) noted that immature (28-day-old) *F. hepatica* synthesized more than 30 biosynthetically (^{35}S-methionine) radio-labelled immunoreactive glycoproteins, of which the major ones released into the culture medium had molecular sizes of 25-38 kDa. These were reactive with sera obtained

from rabbits 3 or 9 weeks after infection with *F. hepatica*. Another major immunoreactive glycoprotein (260 kDa) released into the culture medium was only reactive with sera from rabbits with a 3-week-old infection. In contrast, several synthesized glycoproteins in the molecular size range of 45 to 74 kDa which were not released into the medium were reactive only with sera obtained 9 weeks after infection. Irving and Howell (1982) stated that the total excretory/secretory products of D21 *F. hepatica* biosynthetically radio-labelled using [^{14}C]leucine, [^{14}C]isoleucine or [^{35}S]methionine contained three major polypeptides with apparent molecular weights of 24, 26 and 27 kDa and a less prominent one of 23 kDa. In ^{14}C-leucine and ^{35}S-methionine labelled ES, the 23 kDa band was more pronounced and the 27 kDa band was less intensively labelled. The three major proteins were immunoprecipitated by sera from rabbits and sheep immunised with somatic and ES products respectively (Table 2.1).

Sera from infected rats as well as a monoclonal antibody produced from rats that had been infected with *F. hepatica* detected antigens of 30, 32, 41, 58, 70, 91-94 and 200 kDa in somatic extracts of 3-week-old flukes. Majority of these antigens contained both phosphorylcholine and non-phosphorylcholine epitopes (Sloan, Dooge and Joyce, 1991).

2.4.3 "Antigens" of Adult Fluke

The entire mature stage of liver flukes or extracts derived from them, have been the focal point of many immunological studies. This is most likely due to the ease of recovery of this stage from infected animals.

2.4.3.1 Surface and somatic "antigens" of adult flukes

Hillyer (1980) isolated tegumental antigens of adult *F. hepatica* which gave a positive immunodiffusion reaction with sera from rabbits and humans infected with *F. hepatica*. Fractionation of this antigen by precipitation with ammonium sulphate resulted in a soluble aqueous phase which detected infection in an ELISA or Enzyme linked Immunosorbent Assay in rabbits at 2 weeks after infection

(Hillyer and Serrano, 1986). De Weil, Hillyer and Pacheco (1984) eluted antigens that resulted from reacting tegument extracts of adult *F. hepatica* with a CNBr-sepharose 4B column coupled with IgG from rabbits with a 6-8 week old infection of *F. hepatica*. Fractionation of this eluate on sepharyl S 200 column resulted in three major peaks. Only rabbits immunized with peak 2 (25 kDa) showed strong precipitin lines when reacted against somatic and surface extracts of adult fluke by immunodiffusion and immunoelectrophoresis.

Pfister, Daveau and Ambroise-Thomas (1984) fractionated a homogenate of adult *F. hepatica* on sephadex G200 and observed that the third (Fhs1) peak showed the highest ELISA activity. This fraction detected *F. hepatica* infection in rabbits after 19 days. However, the authors did not give the molecular weight of this fraction. Santiago and Hillyer (1986) fractionated a similar homogenate on sephacryl S-200 and tested sera from infected rabbits, cattle, and humans by western blots against peaks 3 and 4, which included polypeptides in the 14-45 kDa range. Antigens with molecular weights of about 11-14, 18-23, 27-28 and 31-33 kDa were recognised by each of the test sera. The 18-23 kDa antigens were strongly identified by sera from infected cows but only faintly identified by sera from infected humans and rabbits. By use of western blotting, Santiago and Hillyer (1988) found that sera obtained from sheep and cattle 4-6 weeks after infection recognised major polypeptides with molecular weights of 30-38 kDa in somatic extracts of adult *F. hepatica*. Four to five polypeptides with molecular weights ranging from 20 to 25 kDa were detected by sera from infected sheep but only faintly, if at all, by sera from infected bovids. Three clusters of polypeptides with approximate weights of 56, 64, and 69 kDa were recognised by the bovine sera early in infection but were not recognised by the ovine sera. Sloan *et al.* (1991), who probed immunoblots of somatic extracts of adult *F. hepatica* with polyclonal (rat) and monoclonal antibodies, identified antigens with apparent molecular weights of 31, 32, 41, 58, and 160 kDa.

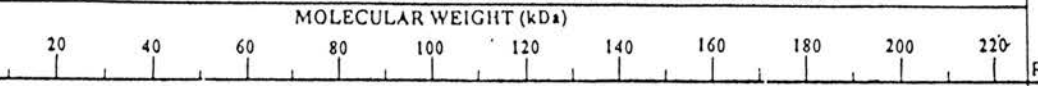
2.4.3.2 Antigens in the ES products of adult fluke

Immunodiffusion reactions between a whole metabolic (ES) antigenic preparation of adult *F. hepatica* with sera from infected sheep, rabbits and rats gave three, one and two precipitin lines respectively (Lehner and Sewell, 1980). Metabolic antigens which had been completely adsorbed with sera from any of the three infected hosts still gave precipitins reaction with sera from the other two host species. Following fractionation of the antigenic preparation, sera from all three species reacted with antigens fractions within 10-25 kDa. Sandeman and Howell (1981) found that antiserum collected from sheep after 8 or 12 weeks of infection formed 5 and 6 precipitin lines respectively in immunodiffusion reactions with ES products from adult flukes.

Sepharyl S200 separation of ES from adult *F. hepatica* resolved four peaks (40, 70, 202 and 376 kDa) while sepharose 6 separation resolved 9 peaks with apparent molecular weights of 2, 3.6, 5.4, 12, 24, 59, 81, 217 and 362 kDa) respectively. Fractions with molecular weights of 24, 40, 59 and 70 kDa were dot-ELISA positive (Zimmerman and Clark, 1986). Rivera-Marrero, Santiago and Hillyer (1988) separated adult fluke ES by gel filtration and eluted two main peaks containing polypeptides with weights of 12-14, 25-48, 150-160 kDa respectively.

Santiago, Hillyer, Garcia-Rosa and Morales (1986) detected by western blotting that sera from rabbits infected with *F. hepatica* for 5-6 weeks identified a cluster of 7 polypeptides with weights of 23-28 kDa in ES from the adult fluke. Five additional polypeptides (39, 52, 58, 84 and 120 kDa) were recognised by sera taken after 6 to 52 weeks of infection. Using this same technique, Hillyer and Soler De Galanes (1988) showed that sera collected from humans, calves, sheep and rabbits infected with *Fasciola hepatica* recognised two antigenic polypeptides of 17 and 63 kDa in the ES of adult fluke. In addition, sera from infected sheep and calves recognised antigens in the "high 20s to low 30s" range.

Table 2.1: COMPONENTS AND ANTIGENIC COMPONENTS OF *FASCIOLA* SPP.



TOOL	FLUKE	REFERENCES
Surface labelling (components)	D0	Lammas and Duffus, 1985.
	D7	
	D14	
	D0	Dalton and Joyce, 1987.
	D21	
Biosynthetic labelling (components)	D0 ES	Ajanusi, 1993.
	D1 ES	
	D14 ES	
	D28 ES	
	D21 ES	Irving and Howell, 1982.
	D28 glycoprotein	Dalton *et al.*, 1985.
	D42 ES	Ajanusi, 1993.
	Adult ES	
	Adult ES	Sexton *et al.*, 1991.
Gel filtration (components)	Adult ES	Zimmerman and Clark, 1986.
	Adult ES	
	Adult ES	Riverra Marrero *et al.*, 1988.
	Adult ES	Lehner and Sewell, 1980.
Silver staining (components)	D0 ES	Ajanusi, 1993.
	D1 ES	
	D14 ES	
	Adult ES	
	Adult somatic	
	Adult NoG extract	
	Adult CTAB extract	
Western blotting (antigens)	D21 extract	Sloan *et al.*, 1991.
	Adult extract	Santiago and Hillyer, 1986.
	Adult extract	Santiago and Hillyer, 1988.
	Adult extract	Sloan *et al.*, 1991.
	Adult extract	Ajanusi, 1993
	Adult NoG extract	
	Adult CTAB extract	
	D0 ES	
	D1 ES	
	D14 ES	
	Adult ES	
	Adult ES	Santiago *et al.*, 1986.
	Adult ES	Hillyer and Soler DE Galanes, 1988
	Adult ES	Solano *et al.*, 1991.
	Adult ES	Poitou, *et al.*, 1992.
Immuneco-Precipitation (antigens)	D0 ES	Ajanusi, 1993.
	D14 ES	
	D28 ES	
	D42 ES	
	Adult ES	
	Adult ES	Sexton *et al.* 1991.
	Adult ES	Santiago *et al.*, 1986.

Solano, Ridley and Minocha (1991) probed ES from adult *F. hepatica* with four monoclonal antibodies produced against adult fluke ES. These monoclonal antibodies detected antigens of 50 and 53; 160 and 180; 66-93; and 29, 32, 45, 50 and 53 kDa respectively.

Poitou, Baeza and Boulard (1992) reported that antigens with molecular weights of about 22.5 and 115 kDa were recognised by immunoblotting of antisera collected at week two from infected rats with ES of adult flukes. They also stated that antigens of about 37.5, 42.5 and 50 kDa were recognised by sera collected from four weeks of infection while antigens with molecular weights of about 33 and 60 were recognised by sera collected from week 5 of infection.

Sexton, Millner and Campbell (1991) biosynthetically radio-labelled ES of adult *F. hepatica* and reported that the dominant molecules had molecular weights of between 29-31 kDa. Immunocoprecipitation revealed that a 29-43 kDa antigen was identified by sera taken from sheep at week 4 after infection. Santiago *et al.*, (1986) revealed that immunocoprecipitation using sera from rabbits 3-5 weeks after *F. hepatica* infection detected antigens of about 33 and 62 kDa in ^{35}S-methionine-labelled ES products of adult *F. hepatica* while sera taken 9 weeks after infection recognised antigens of 38, 40, and 44 kDa.

CONCLUSION

There is abundant evidence on the ability of some hosts, notably the rat and cattle, to develop resistance to challenge following a primary infection. The ability of antiserum from these animals to transfer passive immunity to experimental animals is suggestive of involvement of humoral antibodies in protection. Sera from hosts such as sheep which are incapable of developing resistance after a primary infection have also been shown to confer some degree of protection in passive transfer experiments. However, the nature of this protection has not been characterized at the molecular level.

In addition, the results of several workers have demonstrated that experimental immunization of animals with ES products of various stages or ages of this parasite confers significant protection upon recipients. This suggests that protective serum antibodies may possibly be directed at some ES components. It can be implied therefore that changes in the ES products of the parasite might have important implication in its host/pararite relationship. As of now there is no available report on the sequential study of biosynthetically radio-labelled ES products of *F. hepatica.* Yet, molecular characterization of the parasite's ES products is an important and necessary step towards the identification of possible protective and or diagnostic components.

The study of Poitou, Baeza and Boulard (1992) involved only the unlabelled ES products of the adult fluke. The study by Lammas and Duffus (1985) concentrated mainly on the surface antigens of D0, D7, D14 and adult flukes.

Biosynthetic labelling, unlike surface labelling, affords the opportunity to study proteins which are actively produced *in vitro* by a parasite. Any radio-isotope-labelled components can then be cloned using the mRNA of the particular stage. The only available reports on biosynthetic labelling of *Fasciola hepatica* proteins were those of Santiago *et al.* (1986) and Sexton *et al.* (1991), both of which were done on adult fluke.

This study was therefore designed to characterize ES products of *Fasciola hepatica* as it develops in the rat and to characterise components that might be involved in conferring protection in passive transfer experiments.

CHAPTER THREE

MATERIALS AND METHODS

3.1 RATS

All experiments were conducted on male Wistar rats purchased as specific pathogen (helminth) free animals from Bantin and Kingsman. They were aged five to six weeks at the start of each experiment.

3.1.1 Management

The rats were kept paired in cages with a polypropylene body and stainless steel top, measuring 380 x 220 x 190 mm. The cages contained a mixture of fresh wood shavings (BS&S Ltd, Edinburgh) and Okaite spill-dry (Okaite, Edinburgh) as dessicant. Water and rat and mouse maintenance diet (Labsure) were supplied *ad libitum* and the animals were kept at a temperature of between 21°C and 23°C. The litter was changed three times a week and the lids of the boxes were cleaned every two weeks.

3.1.2 Infection of Rats With Metacercariae

The required numbers of metacercariae were gently scrapped off the polythene bag on which they had encysted using a mounted needle. They were then transferred onto a watch glass containing a few drops of a 1% solution of gum tragacanth (Boots) using a pipette pre-treated with silicone (Replecote^{R}, Hopkin & Williams, England). A silicone-treated plastic tube fitted onto a 2-ml syringe was used to administer the metacercariae directly into the stomach of the rat under light ether (Anaesthetic B.P; May and Baker) anaesthesia.

Three trials were conducted to produce antiserum following the protocol described in Table 3.1.

40 uninfected control rats were included in each trial to obtain normal rat serum.

Table 3.1 Experimental protocol for rats infected to produce antiserum in the three trials

Trial	No of rats	Metacercariae Dose per rat	Length of trial (days)
1	40	10	56
2	40	20	56
3	40	20	56

3.1.3 Blood Sampling From Rats

Infected rats were bled on days 0, 14, 28, 42 and were exsanguinated at 56 days after infection. Rats that were used for passive protection trials 2 and 3 were bled at days 1 and 3 as well.

A small incision was made at the tail of each rat and blood was collected into vials without anticoagulants.

At the end of each experiment rats were exsanguinated. First they were deeply anaesthetized with ether and laid in dorsal recumbency. They were then exsanguinated by external cardiac puncture using a 14G needle. The blood was placed in sterile Universal bottles.

3.1.4 Preparation of Sera

Vials and Universal bottles containing blood were incubated at 37°C for 1 hour to allow formation of the clot. They were then left overnight at 4°C, followed by a 30 min centrifugation at 3000 g and 4°C. The serum from each bottle was carefully decanted into labelled sterile Bijoux bottles, avoiding inclusion of blood clots, while serum in vials were carefully pipetted off into labelled sterile vials.

3.2 HAEMATOLOGY

3.2.1 Packed Red Cell Volume

Blood was collected from the rats' tails into disposable heparinized capillary tubes (Camlab Ltd). The tubes were two-thirds filled and sealed at one end with

Cristaseal (Hawkley, England). The percentage packed red cell volume (PCV) was determined using a microhaematocrit centrifuge (Haemofuge A, Heraeus Sepatech) and reader, the tubes being centrifuged for five minutes at 17,000 g.

3.2.2 Differential Leucocyte Counts

Thin blood smears were made on clean microscope slides (Chance Propper Ltd, England) and air-dried. They were fixed for two minutes in methanol and then stained for 30 minutes in Giemsa's stain (BDH) diluted 1:10 with distilled water. The slides were washed with Giemsa's buffer (BDH) pH 7.2 and allowed to air-dry.

Differential leucocyte count were performed on the stained slides by differentiating a total of 100 cells in successive microscope fields using the x1000 magnification on a Leitz Dialux 22 microscope (Leica UK Ltd)

3.3 SHEEP

Two female cross suffolk sheep were each infected orally with 500 metacercariae of *F. hepatica* at the age of 18 months (on the 6th of september 1989).

They are maintained in conditions that precluded any accidental infections with other helminth parasites and fed routinely on hay and commercial concentrates consisting of sheep nuts and crushed oats (Seafield mill).

3.4 LABORATORY MAINTENANCE OF *F. HEPATICA*

3.4.1 Culture of Green Algae (*Oscillatoria obscura*)

Top soil containing as few stones as possible was collected. It was passed through a 4 mm mesh to remove stones and other debris before being sterilized by autoclaving at 1 kg/cm^2 for 30 min. After cooling, the soil was mixed with 500 ml of a mineral solution (see Appendix 1) described by Sewell (1973).

The mixture was then smoothed into the bottom of a 104 x 178 mm plastic box (Stewart Plastic Products) to a depth of about 15 mm, to give a flat surface on which algae could grow. A small amount of algae from a feeder culture was placed

in the middle of the box and the whole surface gently sprayed with distilled water. The boxes were placed in a warm room at 23°C, about 60 mm beneath three 38W white fluorescent light bulbs (Gro-Lux, Sylvania, Germany). The algal cultures were gently sprayed with water daily.

3.4.2 Culture of Young Snails

Egg masses from adult *Lymnea truncatula* were placed onto a fresh algal culture and kept in the warm room to hatch. After hatching and eventual depletion of the algae, the young snails were carefully collected using tissue forceps and transferred onto a fresh algal culture.

3.4.3 Obtaining Miracidia of *F. hepatica*

Faecal samples were collected by gloved hands directly from the rectum of the two *F. hepatica*-infected sheep. A suspension of the faeces in tap water was made in a clean plastic bowl. The suspension was passed through a nylon coffee strainer having a pore size of 1 mm and then through a sieve of 106 μm aperture into one of 53 μm aperture. The eggs retained in the latter sieve were transferred into 50 ml flasks (Nunclon, Gibco code 1-63371A) and incubated for five days at 23°C; being washed daily with distilled water. The bottles were wrapped in aluminium foil and kept in the dark for nine days. Miracidia were obtained by decanting the embryonated eggs in the flasks into a petri-dish and exposing the eggs to light.

3.4.4 Infection of Snails with Miracidia

Infection of *Lymnea truncatula* was done as described by Haroun (1979). The snails were infected when they were 2 to 5 mm in length. Seven to ten miracidia were pipetted into each of 50 wells of a flat-bottomed polystyrene microwell plate (Gibco, code 2-62162A) containing 96 (317 μl) wells. The snails were then placed individually into each of the 50 wells. The plates were then covered and left for 4 hours at room temperature, after which the infected snails were transferred onto a fresh algal culture. The infected snails were maintained at

23°C, being transferred to fresh algal plates as the need arose. Five weeks after infection the snails were examined under a stereomicroscope to determine if they contained *F. hepatica* rediae. Snails that did not contain rediae were separated and re-examined after two weeks and those showing no evidence of infection at that time were kept as egg-laying adults.

3.4.5 Shedding of Cercariae and Storage of Metacercariae

50 snails harbouring mature (6-week or more) *F. hepatica* infections were placed in small (120 x 75 mm) polythene bag containing 40 ml of cold (4°C) distilled water. The polythene bag was placed in a bottle and left at room temperature. The cercariae were shed as the water warmed up to room temperature. The snails were removed from the water after about 4 hours and placed on a new algal plate. The bag containing the cercariae was left at room temperature for 3 days, after which the water was poured off, leaving a few drops in the bag. The bag was kept in the fridge at 4°C for a maximum period of 3 months until needed. The discarded water was autoclaved in order to kill any metacercariae that may be present.

Metacercariae were also purchased from the Central Veterinary Laboratory (Weybridge) and from Compton Paddock Laboratory (Newsbury, Berks).

3.5 RECOVERY AND STORAGE OF *F. HEPATICA* FROM INFECTED RATS

3.5.1 Newly Excysted Juveniles (D0)

Metacercariae were excysted using the method described by Lehner (1977). On each occassion 1000 metacercariae were gently scraped off the cellophane bag on which they had encysted. They were pipetted into a sterile petri dish and 25 ml of a solution of 0.8% w/v NaCl, 0.5% w/v pepsin in 0.05N HCl was added. This was followed by incubation for 2 hours at 37°C, after which an equal volume of an aqueous solution containing 1.0% w/v $NaHCO_3$, 0.8% w/v NaCl, 0.4% w/v trypsin and 20% v/v ox bile was added; this being followed by a further 3 hour incubation

at 37°C. At the end of the incubation period the petri dish was examined under a stereomicroscope and, the excysted flukes were pipetted into sterile Universal bottles containing phosphate buffered saline pH 7.4 (PBS, Sigma, code P 4417). The bottles were kept in a water bath at 37°C for 20 min to allow the flukes to sediment. The supernatant was decanted, leaving about 2 ml of fluid which was then transferred into Eppendorf tubes. The tubes were centrifuged at 22 g for 1 min, after which the supernatant was pipetted off, leaving the flukes in about 100 µl of sterile phosphate buffered saline.

3.5.2 Invasive Stages (D1 flukes)

To recover this stage, three rats were each orally infected with 500 metacercariae. The rats were killed 24 hours after infection and invading flukes were recovered using the method described by Davies and Goose (1981). Ten ml of warm (37°C) sterile phosphate buffered saline (PBS) was injected into the peritoneal cavity. A mid-line longitudinal incision was made on the skin from the promontory of the pelvis to the thoracic inlet. Another incision was made horizontally along the long axis of the last rib and a third incision was made along the brim of the pelvis, so that skin flaps were formed. The fascia, muscle and peritoneum were then cut to expose the viscera. The fluid in the peritoneal cavity was aspirated into sterile Universal bottles with sterile syringes. The flukes were then concentrated by sedimentation.

3.5.3 Parenchymal or Migratory Stages (D14, D28 and D42).

These stages were recovered from the liver using the method described by Harness, Doy and Hughes (1977). Three rats were each orally infected with 150 metacercariae. They were killed 14, 28 or 42 days after infection and their viscera exposed as described above. The liver was freed of all its adhesions and was transferred into a petri-dish containing sterile phosphate buffered saline (PBS, Sigma, code P 4417) at 37°C. It was then broken up into small (10 mm) pieces by applying slight pressure with a gloved thumb. The liver suspension was then

incubated at 37°C for 4 to 6 hours, with periodic examination under a stereomicroscope for emerging flukes. When a fluke was observed it was pipetted into a Universal bottle containing warm (37°C) sterile Phosphate buffered saline (PBS, Sigma code P4417) and kept in a waterbath at 37°C. After the 6 hour incubation by which time about 80 flukes would have been recovered, the flukes were washed several times with sterile Phosphate Buffered saline (PBS) under a sterile hood.

3.5.4 Adult Flukes

Rats were each orally infected with 10 or 20 metacercariae and at D56 of infection they were deeply anaethetized with ether and exsanguinated. The liver was exposed as described above. The common bile duct was located, clamped with artery forceps at its junction with the duodenum and severed. The liver was lifted and the flukes were then gently expressed from the bile ducts.

Flukes that were not needed for immediate use were washed six times in phosphate buffered saline, packed into 1 ml cryovials (BDH, 215/0880/02) and preserved in liquid nitrogen at -196°C .

3.6 HISTOLOGY

3.6.1 Preparation of Histological Section

Sections of liver were fixed in 10% v/v formalin immediately after removal from the animal. The fixed samples were trimmed, embedded in paraffin wax and cut into thin 4 μm sections with a microtome by standard methods. Paraffin sections were stained with haematoxylin and eosin (H&E) stain (Bancroft and Stevens, 1977)

Sections to be differentially stained for eosinophils were washed in water for 30 min and then for 1 min in Harris haematoxylin (Sigma, HHS-16). They were again washed in Scott's tap water substrate (170 mM $MgSO_4$ and 40 mM $NaHCO_3$ in distilled water) and then stained for 10 min in Carbol-Chromatrope (10 mM

Carbol Chromatrope 2R and 100 mM Phenol in distilled water), after which they were dehydrated in alcohol, cleared in xylene and mounted in synthetic resin medium (Neil MacIntyre, personal communication).

3.6.2 Scanning Electron Microscopy

Scanning electron microscopy were done by the EM Unit of the Faculty.

Flukes were fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.3 for 2½ h at 4°C. They were then washed in three changes (20 min each) of 0.1M sodium cacodylate buffer followed by fixation for 45 min in the buffer, after which the samples were washed (3 x 20 min) in distilled water. They were dehydrated through a series ascending concentration (50, 70, 90 and 100%) of acetone, each step lasting for 20 min and dried in CO_2 using the critical drying apparatus (series 3000, Polaron, Hertfordshire).

The dried samples were then mounted on aluminium stub using Liet-C conductive carbon cement (Neubauer, Germany), dried in an oven for 1-2 hours before being sputter coated in an EMscope sputter coater SC 500 with 20 nm gold/palladium mixture. The coated samples were then viewed and photographed on SEM 505 Electron microscope using an FP4 black and white 35 mm (Ilford) film (Stephen Mitchell, EM Unit, personal communication).

3.7 PASSIVE PROTECTION TRIALS

Three groups of rats were used in each trial and the experimental protocol is as described in the Table 3.2. A total of 24 rats were used in the first trial while a total of 30 rats were used in the two later trials. The rats were bled before infection and then at fortnightly intervals but in trials 2 and 3 rats were also bled at D1 and D3 of infection. The rats were exsanguinated at D56 for determination of fluke burdens. Fluke burdens of groups were compared using Mann-Whitney test.

Table 3.2 Experimental protocol for passive protection studies

Group	Treatment	Challenge dose/rat (metacercariae)
1	10 ml of antiserum given IP at challenge and 48h after	20
2	10 ml of normal rat serum IP at challenge and 48h after	20
3	No serum given	20

3.8 PREPARATION OF PARASITE EXTRACTS

3.8.1 Somatic Extracts

One ml of packed adult *F. hepatica* was removed from storage in liquid nitrogen and pulverized into a powder using a pestle and mortar on dry ice. The powder was transferred into 4 ml of a solution of PBS containing protease inhibitors (PBS-PI, see Appendix 2). The mixture was left on ice for 15 min with occasional shaking, after which it was transferred to sterile Eppendorf tubes and centrifuged for 20 min at 14000 g at 4°C. The supernatant was pooled and its protein content was determined as described by Warburg and Christian (1941). This extract was aliquoted in 200 µl volumes and stored at -70°C until needed.

3.8.2 Cetyltrimethyl-ammoniumbromide (CTAB) and N-octyl Glucopyranoside (NOG) Extraction

Freshly collected adult flukes were washed were washed six times in sterile PBS. Eight flukes were transferred to Bijoux bottle containing 2 ml of 0.25% w/v solution of CTAB or 1% w/v solution of NOG in PBS-PI and left on ice for 30 min with occasional shaking.

The extracts were transferred to dialysis tube (Sigma) and dialysed overnight against sterile PBS so that the resultant concentration of the detergent was 0.03% w/v. The protein content of the extracts were determined before storage at -70°C in 150 µl aliquots.

3.9 COLLECTION OF TOTAL EXCRETORY/SECRETORY PRODUCTS

3.9.1 From D0 and D1 Flukes

After recovery, about 200 newly excysted juvenile flukes contained in 100 µl of phosphate buffered saline in an Eppendorf tube, were transferred into a sterile Universal bottle containing 25 ml of warm (37°C) RPMI 1640 (Gibco Ltd) in a 37°C water bath. They were allowed to sediment for 10 min, after which they were gently pipetted into another Universal bottle containing 25 ml of RPMI 1640 for another wash. Using this procedure, the flukes were given two more washes in the complete culture medium (see Appendix 2). After the last wash, they were pipetted into a sterile Eppendorf tube and centrifuged at 22 g for 1 min at room temperature. The supernatant was carefully pipetted off, leaving the flukes in about 100 µl of culture medium. They were then pipetted into one well of a 24-well tissue culture plate (Nunclon, Gibco) containing 400 µl of complete culture medium. They were incubated for 24 hour at 37°C in an atmosphere of 5% v/v CO_2 in air. The culture supernatant was collected and centrifuged at 358 g for 10 min. The supernatant obtained was again centrifuged for 30 minutes at 12000 g and 4°C. The protein content of the culture supernatant was determined (as described by Warburg and Christian, 1941), as was the protein content of the complete culture medium. The difference between these two values was taken to be the amount of the protein in the preparation derived from the parasites' excretory/secretory products. The ES from newly excysted juvenile (ES_0) or one-day-old flukes (ES_1) was then stored in 150 µl aliquots at -70°C until needed for silver staining or western blot analysis.

3.9.2 From Parenchymal Flukes (D14).

After recovery the 14-day-old flukes were washed six times with sterile (37°C) Phosphate buffered saline (PBS), followed by six washes in pre-warmed (37°C) RPMI 1640 (GIBCO) and a further six washes in the pre-warmed (37°C) complete culture medium in a sterile hood. The cleaned flukes were then transferred into a sterile petri dish (Nunclon, Gibco, I-68381A) containing 20 ml of warm

(37°C) complete culture medium. Using a sterile glass pipette, 75 flukes were transferred into one well of a 24-well tissue culture plate (Nunclon, Gibco code 1-43983A) containing 2 ml of complete culture medium. The surrounding wells were each filled with 1 ml of RPMI 1640 to reduce evaporation. The plate was incubated at 37°C for 24 hours, after which the culture supernatant was collected and centrifuged at 14000 g for 30 min. The protein content was determined as described by Warburg and Christian (1941) and the ES (ES_{14}) stored at -70°C.

3.9.3 From Adult Flukes

After recovery from the bile ducts, the flukes were washed six times in sterile phosphate buffered saline, followed by six washes in pre-warmed RPMI 1640 (Gibco) and a further six washes in complete culture medium.

Active undamaged flukes were identified, and one fluke each was transferred into 8 adjacent wells of a 24-well tissue culture plate (Nunclon, Gibco), each of which contained 1 ml of complete culture medium. One ml of RPMI 1640 was pipetted into each of the unused wells surrounding the fluke-containing wells to reduce evaporation. The plate was then covered and incubated for 24 hours in an atmosphere of 5% v/v CO_2 in air at 37°C. The culture supernatants from the fluke-containing wells were then pooled, aliquoted into Eppendorf tubes and centrifuged at 1000 g for 5 min at 4°C. The supernatants were again pooled and centrifuged at 14000 g for 30 min at 4°C. The protein content was determined as described above.

3.10 BIOSYNTHETIC RADIO-LABELLING OF *F. HEPATICA*

3.10.1 Radio-Labelling of Parasites and Collection of Radio-labelled Parasite Products

This procedure was performed as described by Joshua, Harrison and Sewell (1988).

D0 and D1 Flukes

About 200 newly excysted juveniles (recovered as described in 3.5.1.) or day-old flukes (obtained as in Section 3.5.2) contained in 100 µl of sterile phosphate buffered saline were washed once in complete culture medium and once in methionine-free medium (Selectamine, Gibco) prepared as instructed by the manufacturers. The flukes contained in about 100 µl of methionine-free medium were then transferred to a well of a 24-well (3.14 ml well volume) tissue culture plate (Nunclon, Gibco) containing 2 ml of the methionine-free medium. 7.4MBq of ^{35}S-methionine (1000 ci/mMol, Amersham code SJ 1015) was pipetted into the well. The plate was labelled, covered and placed on a box of charcoal (to absorb any radioactivity) and cultured for 24 hours at 37°C in an atmosphere of 5% v/v CO_2 in air.

At 24 hours, the culture supernatant containing any radio-labelled parasite excretory/secretory products was collected into a sterile Bijoux bottle on ice. Non-radioactive L-methionine (50 µg/ml) (Sigma, code M9625) was added to the supernatant, after which it was centrifuged for 5 minutes at 1000 g and 4°C. The resulting supernatant was again centrifuged for 30 minutes at 14000 g and 4°C. The supernatant was collected into sterile Bijoux bottles and desalted.

D14, D28 and D42 Flukes

About 70-80 D14, 40 D28 or D42 flukes recovered as described in 3.5.3 were, under a hood, washed in 30 ml of complete culture medium contained in 144 x 21 mm sterile polystyrene petri dish (Nunclon, Gibco code 1-68381A). The flukes were transferred into, and washed in 30 ml of methionine-free medium contained in a similar petri dish. The flukes were then transferred into 2 ml of methionine-free medium (Selectamine, Gibco) in a well of a 24-well tissue culture plate (Nunclon, Gibco). One ml of complete culture medium was pipetted into the surrounding wells to reduce evaporation. 7.4MBq of ^{35}S-methionine (1000 ci/mMol, Amersham code

SJ 1015) was pipetted into the fluke-containing well. The plate was placed on a box of charcoal to absorb any radioactivity and then cultured for 24 h at 37°C in an atmosphere of 5% v/v CO_2 in air. After 24 h, the culture supernatant was collected and treated as already described while the flukes were washed in phosphate buffered saline containing protease inhibitors before storage in liquid nitrogen.

Adult Flukes

Recovered adult flukes (3.5.4) were kept for 1 hour in an incubator at 37°C to allow them regurgitate their caecal contents. Under a hood, the flukes were given six washes in sterile phosphate bufferred saline, followed by six washes each in RPMI 1640 (Gibco), complete culture medium and methionine-free medium respectively.

Active undamaged flukes were identified and one of such flukes was transferred to each of six wells of a 24-well tissue culture plate (Nunclon, Gibco) containing 1 ml of methionine-free medium (Selectamine, Gibco). 1 ml of culture medium was pipetted into each of the adjacent wells. 1.85MBq of ^{35}S-methionine (activity 1000 ci/mMol, Amersham, code SJ 1015) was added to each of the fluke-containing wells. The plate was then covered, placed on a box of charcoal and incubated for 24 hours at 37°C in an atmosphere of 5% v/v CO_2 in air. After incubation, the labelled flukes were washed in phosphate bufferred saline containing protease inhibitors and stored in liquid nitrogen. The culture supernatant in all the fluke-containing wells was collected, pooled and centrifuged for 10 minutes at 500 g and 4°C. The resulting supernatant was again pooled and centrifuged for 30 minutes at 14000 g and 4°C, after which the supernatant was pooled and desalted.

3.10.2 Preparation of Somatic Extracts of Flukes

Somatic extracts of flukes were prepared as described by Joshua *et al.* (1988).

The flukes were recovered from liquid nitrogen and homogenised on dry ice in sterile mortar and pestle. The powder was transferred to an Eppendorf tube containing 0.95 ml of PBS-PI, and 50 µl of Nonidet P40 was added to the homogenate, which was then held on ice for 30 minutes. The homogenate was then centrifuged at 14,000 g for 3 minutes at room temperature after which the supernatant was desalted.

3.10.3 Desalting the Supernatant

For every 2 ml of supernatant to be desalted 20-ml bed volume of Sephadex G25 was prepared. The approximate weight of fine sephadex G25 (Pharmacia) required to give the desired volume was swollen in phosphate buffered saline overnight.

The gel was poured into Bio-Rad columns, and packed down with PBS-PI-Methionine (PBS-PI containing 0.67 mM L-methionine [Sigma, m9625]) to the 20-ml mark, ensuring that the surface of the gel was uniformly flat.

Each column was washed once with PBS-PI-Methionine. The PBS-PI-Methionine was drained down to the top of the column and 2 ml of the radio-labelled ES product was immediately added and run into the column. The ES was then eluted with PBS-PI-Methionine. Two 2-ml fractions and 34 0.5-ml fractions were collected from each column into pre-labelled tubes.

Thirty six scintillation vials were numbered and serially arranged in racks. Then, 4 ml of scintillation fluid was dispensed into each. Starting from the first fraction, 10 µl from each eluate was sequentially added to the respective vials. The vials were covered and the activity in each was counted on a scintillation counter (Parkard) using the programme for ^{35}Sulphur. The fractions containing the radio-labelled parasite excretory/secretory products (i.e, peak 1) were pooled.

3.10.4 Trichloroacetic Acid Precipitation

From the pooled fractions 10 µl was pipetted into each of four test tubes. Then 190 µl of 0.15M NaOH was added to tubes 1 and 2.

190 µl of normal rabbit serum diluted 1:10 in normal saline was added to tubes 3 and 4. These two tubes were filled with 10% solution of trichloroacetic acid and centrifuged for 5 min at 2500 g at 4°C. The precipitate in each tube was resuspended in 200 µl of 0.15M NaOH. The tubes were again filled with 10% trichloroacetic acid and centrifuged. The supernatant was gently decanted and each precipitate was resuspended in 200 µl of 0.15M NaOH.

The 200 µl from each tube was pipetted onto a vial containing 4 ml of scintillation fluid. The four vials were counted along with four blank vials containing only scintillation fluid.

The trichloroacetic acid precipitable counts per minute per µl were calculated using this formula:

$[(s/t \times 100)xt]/10$

where,

t = The total count represented by the mean of tubes 1 and 2 after subtraction of the mean of the blank tubes (5-8).

s = the mean precipitable count represented by the mean of tubes 3 and 4 after subtration of the mean of the blank tubes.

All counts were expressed as TCA precipitable counts per µl/minute.

3.11 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

General Protocol

ELISA assays were performed as described by Voller, Bidwell and Barlett (1979) to monitor antibody responses of rats to infection. Flat bottom (96-well) polystyrene microELISA plates (Immunolon, Dynatech Laboratories Inc. Virginia) were coated with 50 µl/well of Excretory/Secretory or somatic antigens of adult fluke, each antigen having been diluted to the optimal dilution of 1 µg/ml in Borate Buffered saline (99.95 mM boric acid, 47.42 mM disodium tetraborate and 74.95 mM sodium chloride, pH 8.2). The plates were wrapped with cling film to

prevent evaporation and incubated overnight at 4°C. The plates were washed twice with a solution of 0.15M NaCl containing 0.02% w/v Tween 20 (Sigma code P 1379), allowing each wash to stand for 3 min then dried by shaking onto a pad of paper towels. Then, 100 µl of diluent made of 4% v/v normal goat serum (NGS, Gibco) in PBS containing 0.02% v/v of Tween 20 (NGS/PBS/Tween) was added to each of the well and the plates were left for one hour at room temperature to block any non-specific reaction sites. After shaking out the diluent and washing each plate three times, 50 µl of test serum sample appropriately (1:200) diluted was added to each well. Each test serum was run in duplicate. Samples of similarly diluted sera from known infected and non-infected rats were each included as controls in five wells of each plate.

The plates were covered with cling film and incubated for 1 hour at 37°C.

Following the wash procedure as described above, 50 µl of enzyme conjugate (Goat anti-rat (heavy and light chain) IgG/Peroxidase [GAR/PO] whole molecule, Nordic Immunological Laboratories, The Netherlands) diluted to the optimal concentration of 1:1000 in the diluent, was added to each well. The plate was then incubated for a further 1 hour at 37°C, then washed.

Substrate at 50 µl/well containing 0.42 mM N,N,N',N'-Tetramethylbenzidine (Sigma code T 5513) in (dimethylsulphoxide ,Sigma) diluted in 100 mM sodium acetate/citric acid buffer (pH 8.0) containing 0.13 mM urea hydrogen peroxide (Sigma code U 1753) was added at room temperature. The colour reaction was stopped after 10 to 15 minutes by the addition of 50 µl of 0.02M H_2SO_4 (Aristar, BDH) to each well and the optical density of the reaction product was measured at 450 nm using a Titertek Multiscan (Labsystems).

Corrected absorbance was calculated:-$[(P_1-N_1)/(P_n-N_n)] \times T_n$

where, P_1 = mean of five replicates of control positive serum assayed on day 1 in plate 1.

N_1 = mean of five replicates of control negative serum assayed on day 1 in plate 1.

P_n = mean of five replicates of control positive serum assayed on day n in plate n.

N_n = mean of five replicates of control negative serum assayed on day n in plate n.

T_n = mean of one duplicate of test serum assayed on day n in plate n.

3.12. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoresis was conducted using the discontinous system described by Laemmli, 1970.

3.12.1 Large Gels

Electrophoresis of large gels were carried out using the Gel Electrophoresis apparatus GE-2/4 LS (Pharmacia) and following the manufacturers' instruction.

The stock solutions (Appendix 2) were mixed to prepare the required gradient gel solutions, 10 - 20% in most cases. Samples were solubilized in reducing or non-reducing buffers (Appendix 2) in a 2:1 ratio and heated for 5 minutes at 100°C. Samples intended for western blots were loaded at 0.595 μg/cm^2 of gel while samples intended for silver staining were loaded at 10 μg per track. The samples, along with prestained molecular weight markers (Sigma, code P1677) were then electrophoresed at 150V and 0.6A using the power pack model 1000/500 (Bio-Rad) as source of electricity. After the samples had entered the resolving gel, electrophoresis was continued for 3-4 hours, during which the system was cooled by the Multitemp LKB apparatus set at 8°C. At the completion of electrophoresis the gels were removed as instructed in the Pharmacia manual.

3.12.2 Minigels

These were also run using the discontinous buffer system (Laemmli, 1970).

Samples were electrophoresed in the Mini-Protean^{R} II Dual Slab Cell apparatus (Bio-rad) following the manufacturer's instructions.

A 12% homogenous gel solution was prepared (Appendix 2) and the solubilized samples along with prestained markers were electrophoresed for 45 minutes at 200V and 45 mA using the 200/0.2 power supply (Bio-rad). Samples for western blotting were loaded at 0.272 μg (ES_{D0}) and 0.15 μg (ES_{D1}) per cm^2 of gel respectively.

3.13 SILVER STAINING OF GELS

Silver staining of gels was done as described by Morrisey (1981).

After electrophoresis, gels were placed individually in suitable clean glass pyrex trays and fixed for 30 min on an orbital shaker (KL2 Edmund Buhler Tubingen) in a solution of 50% v/v methanol and 10% v/v glacial acetic acid in distilled water. After 30 min this solution was replaced by a solution containing 5% v/v methanol and 7% v/v glacial acetic acid. Then after a further 30 min by a solution containing 10% v/v glutaraldehyde using 6 ml per gel. The gels were then soaked in distilled water overnight, and then soaked for 30 minutes in a solution (5 μg per ml of water) of dithiothreitol using 12 ml per gel. The gels were then exposed to a solution of 0.1% w/v silver nitrate for 30 min. The staining solution was poured off and the gels were rinsed with the developer, a 3% w/v solution of sodium carbonate to which was added 50 μl of formaldehyde. The gels were then left in 100 ml of fresh developer solution until the staining was optimal, after which the reaction was stopped by the addition of 10 ml of 2.3 M citric acid. After a further 30 min the solution was poured off and replaced with distilled water until the gel was photographed.

3.14 BLOTTING

3.14.1 Dot Blot

Dot blots were performed using Bio-Dot Microfiltration Apparatus (Bio-Rad) following the manufacturers instruction.

3.14.2 Western Blotting

3.14.2.1 Electrophoretic transfer of proteins

The procedure was performed following the description of Towbin, Staehelin and Gordon (1979).

Following separation of proteins on gradient gel as described in 3.12, the gels were soaked in Bjerrum and Schafer-Nielsen transfer buffer (see Appendix 2)

The proteins were transferred onto nitrocellulose membrane with a pore size of 45 μm (Schleicher and Schuell, W. Germany) using the Trans-Blot^{R} SD Semi-Dry Electrophoretic Transfer Cell (Bio-rad) and following the instructions of the manufacturers. Large gels were transferred for 45 minutes at 10V and a current limit of 0.76A per gel, while minigels were transferred at the same voltage but a current limit of 0.22A per gel.

After transfer, the NC was then placed in a blocking buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.05% Nonidet P40 and 0.25% gelatin in distilled water (see Appendix 2) overnight. It was given three washes of ten min each in 30 ml of 0.15M NaCl/0.02% (w/v) Tween 20, following which it was dried between two filter papers. The well-forming comb was used to indicate the position of the sample tracks and of the markers on the NC membrane. Both ends of the part of the NC membrane containing the blotted proteins were marked at 5 mm intervals with a ball-point pen and the NC membrane was cut into 5 mm vertical strips.

3.14.2.2 Reaction of strips with sera

NC membrane strips which were to be reacted with test sera were incubated overnight in a solution of 4% v/v NGS in blocking buffer. Next day they were given

3 x 10 min washes in 0.15M NaCl/0.02% (w/v) Tween 20 (saline/Tween 20) on an orbital shaker. The strips were then placed in slotted trays (Bio-rad western blot tray) and 5 ml of appropriate test serum diluted 1:200 in 4% NGS (v/v)/blocking buffer was pipetted into the corresponding slots, noteing each strip's number and the type of serum being tested in it. The tray was rocked for 30 min at 37°C in an incubator (Lab-therm, Adolf Kuhner AG Schweiz), after which the strips were given three ten-min washes in saline/Tween 20. They were incubated for 30 min in 25 ml of biotinylated goat-anti-rat IgG conjugate (Sigma) diluted to the optimum concentration in 4% NGS (v/v)/blocking buffer and then washed three times in saline/Tween 20. The strips were again incubated for 30 min in 25 ml of streptavidin alkaline phosphatase conjugate (Amersham) diluted to optimal concentration in 4% NGS/blocking buffer. After further washing, colour development was achieved by incubating the strips in the substrate, 0.1 M diethanolamine containing 5 mM $MgCl$, 0.4 mM nitroblue tetrazolium (in 70% dimethylformamide) and 0.44 mM bromo-chloro-indolyl-phosphate (see Appendix 2). When colour development was judged to be optimal, the developing solution was poured off and the strips were either photographed or dried between filter papers and kept in a dark place until they were photographed.

3.15 IMMUNO-COPRECIPITATION

This procedure was carried out as described in laboratory notes of helminthology section (CTVM).

Normal rat serum, goat-anti-rat gamma globulin and co-precipitation diluent (20 mM Tris-HCl buffer pH 8.0, containing 50 mM NaCl and (0.2% v/v) Nonidet P40) were mixed in a ratio of 1:20:50 and left overnight at 4°C. The volume of radio-labelled ES required for the test sera (at 50,000-100,000 cpm/ per sample) was added to an equal volume of the overnight precipitate. The mixture was left on ice

for 5 min and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was gently pipetted off and added to an equal volume of diluent.

The volume of this antigen required for immuno-coprecipitation with test sera was calculated using this formula :

$$[(mt/v)/P] \times n$$

where,

P = cpm/µl required for each test serum.

m = volume of radio-labelled ES added in the first instance.

t = TCA precipitable count of the radio-labelled ES

v = the final volume in which the labelled antigen was contained.

n = number of test sera to be used.

3.15.1 Immuno-coprecipitation with Test Sera

This was done as described by Joshua *et al.* (1988).

After pipetting 5 µl of relevant serum into two labelled test tubes, the required volume of the prepared antigen was added to each test tube, mixed then left for 1 h at 4°C. Goat-anti-rat gamma globulin (which had earlier been titrated to determine the minimum volume required to give visible precipitate when reacted with 5 µl of serum and 1 ml of diluent) was diluted in co-precipitation diluent and an excess of this (400 µl) was added to each tube. The mixture was then incubated overnight at 4°C and 1000 µl of ice-cold diluent was added to the precipitate in each tube. The mixture was vortexed and centrifuged for 10 min at 800 g at 4°C. The supernatant in each tube was aspirated off without disturbing the precipitate. The precipitate was vortexed in 2 drops of diluent and the washing process repeated twice. After the final washing, each precipitate was suspended in 25 µl of diluent.

From the suspension in each tube, 5 µl was pipetted into a scintillation vial containing 4 ml of scintillation fluid for counting of radioactivity of the precipitates.

Pairs of tubes containing the same serum samples were solubilized in reducing and non-reducing sample buffers (see Appendix 2), and heated for 5 min at

100°C. The samples were then subjected to the SDS-PAGE electrophoresis. ^{14}C rainbow molecular weight markers (Amersham) were electrophoresed along with the samples.

Fluorography

This procedure was performed as described by Hames and Rickwood (1990).

After separation of the labelled proteins by SDS-PAGE, the gels were fixed for 30 min in 10% methanol and 5% acetic acid. They were then given two 30 min washes in pure dimethylsulfoxide (Sigma). This was followed by a three-hour washing in a 27.5% w/v solution of 2,5-diphenyloxazole scintran (PPO, BDH) in dimethylsulfoxide (Sigma). The gel was rinsed in cold tap water and left overnight in tap water, before it was dried.

Gel Drying

The gel was soaked for 30 min in 1% v/v glycerol and 10% v/v acetic acid in distilled water.

The methanol trap of the gel slab dryer (Elnor) was half-filled with methanol, which was cooled by the addition of dry ice until the methanol was saturated.

An 18 x 34 cm cellophane membrane backing (Bio-rad) was soaked in distilled water in a tray. The pre-soaked gel was placed on the membrane and the excess membrane was cut off. The gel and the membrane were placed on the gel dryer, and the gel was covered with cling film (Fisions scientific equipment). The mylar sheet was placed on top of the gel and covered with the silicon flap.

The vacuum pump was switched on, holding the silicon flap down until a good seal was produced. The gel dryer timer/heater was set at 45 min. When drying was completed, the vacuum was released slowly. The gel was removed and taped onto a filter paper which has been pre-cut to the size of an autoradiography cassette.

Autoradiography

This procedure was performed as described by Hames and Rickwood (1990).

Aluminium plates used as autoradiography cassettes were made by the workshop unit of the Veterinary Field Station. The 24 x18 cm plates were taped in pairs with an intensifying screen fastened to the inner side of one of the plates. Working in a dark room, the dried gel on the filter paper was placed between the aluminium plates with the gel facing the intensifying screen. An 18 by 24 cm X-ray film (Agfa Curix, Agfa Gevaert N.V. B-2640, Belgium) was placed between the gel and the intensifying screen, with a mark on the film to show the orientation of the gel. The two plates were then taped together, wrapped in a black bag, sealed, placed in a padded envelope and kept at -70°C until it was judged from experience to be ready for development.

Developing the Autoradiographs

The envelope was removed from the freezer and allowed to warm up to room temperature in the dark room. The film was removed from the cassette and developed in a solution of D-19 developer (Sigma code P 5670). The tray was gently but continuously agitated during the development. After 4 min the film was removed and immersed for 4 min in a fixative (H.A. West (X-Ray) Ltd). After fixing, the film was washed in cold tap water for 2 hours, after which it was air-dried and photographed.

3.16 PHOTOGRAPHY

Photography was done using a Polaroid MP 4 Land camera (Polaroid Corp., U. S. A) and type 55 polaroid film as recommended by the manufacturers.

Different types of samples were photographed using various settings (Bob Munro, Personal communication) as indicated in the following table.

Table 3.3 Camera settings for photograghing various samples

Gel type	Camera settings				
	Column height	Billows extension	Aperture (*f* No)	Exposure time (Sec)	Wratten Filter no
Silvers (mini)	16.5	72	11	5	45
Silvers (large)	67.2	23	11	7-8	45
Dot-blot	54.8	20.4	22	2	11
W/blots (mini)	18	65	22	1	16
W/blots (large)	60.5	16	16	2	23
Autorads	75	15.5	16	5	61

3.17 STATISTICS

Non-parametric analysis (Fowler and Cohen, 1990) was used to analyse data sets with small sizes. Analysis of data sets with large sizes was carried out with Instat software (GraphPAD Software Inc. USA).

CHAPTER FOUR

RESULTS

4.1 PATHOLOGICAL CHANGES IN INFECTED RATS

Pathological changes induced by infection was monitored by visual assessment of pathology in the livers of rats that were infected to obtain D1, D14, D28 and D42 flukes. Changes at day 56 of infection were studied by observations on rats used for producing antisera as well as challenge control rats in the passive protection trials which terminated at D56.

4.1.1 Gross Pathology

No liver lesions were observable at day 1 of infection. By D14, however, all the 9 livers examined were markedly friable and enlarged. Seven of these had widespread necrotic tracts as well as fibrinous adhesions to adjacent organs (Figure 4.1). The three livers examined at D28 were all enlarged and had the necrotic lesions seen at D14. The three livers examined at D42 were fibrotic and two had necrotic foci which were confined to one lobe of the liver. In addition, the livers all had fibrinous adhesions to the adjacent organs. A total of 136 livers were examined at D56. All of these appeared fibrotic. There were marked distension of the bile duct wall (Figure 4.2) and gall bladder in all the livers in which flukes were recovered. Apart from this fibrosis, 13 (9.5%) of the livers appeared grossly normal, while a total of 123 (90.4%) showed varying degrees of necrotic lesions. These lesions were only slight in 58 (47%) of the rats, in which the necrotic foci were very few and confined to just one lobe. The lesions were moderately severe and more widespread in 49 (39%) of the rats but very severe in the remaining 16 (13%) rats. In this latter group of rats the bile was very viscous.

4.1.2 Histopathological Changes

All the 9 livers examined at D14 as well as the three livers examined at D28 showed cellular infiltration of the parenchyma (Figure 4.3a), the main cell involved in this reaction being eosinophils (Figure 4.3b). Hepatocytes in all the sections examined showed degeneration with cloudy swelling. The three liver sections from rats with 28-day-old infection also had mild interlobular fibrosis.

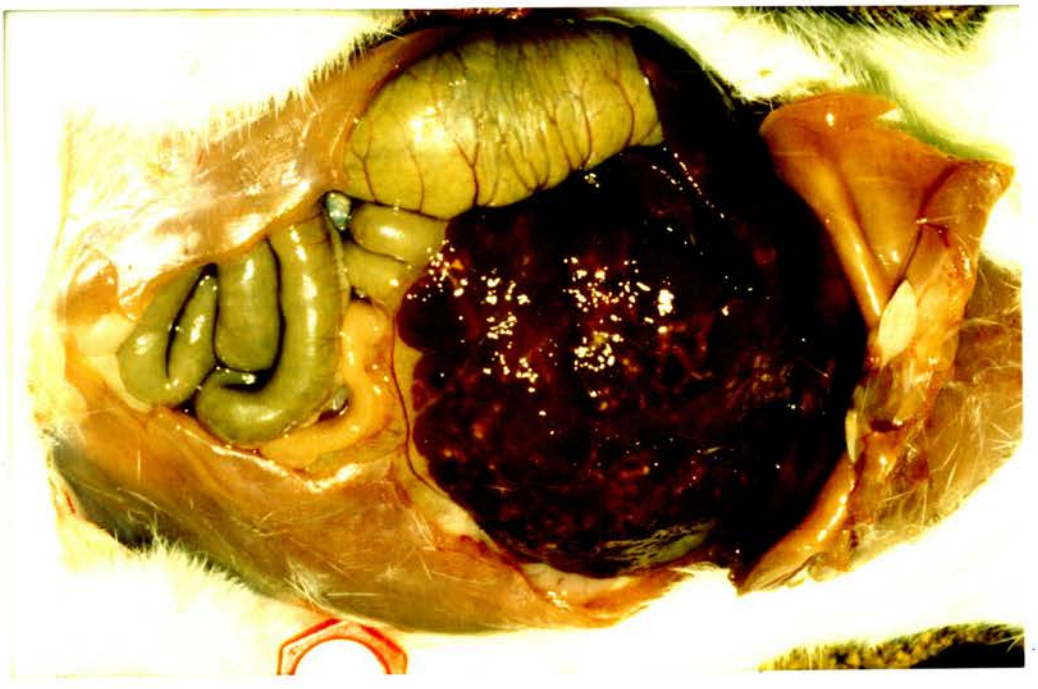


Figure 4.1.

Necrotic tracts on liver recovered at D14 from a rat infected with 20 *F. hepatica* metacercariae.

Figure 4.2

Liver recovered at D56 from a rat infected with 20 *F. hepatica* metacercariae showing enlargement of the bile duct.

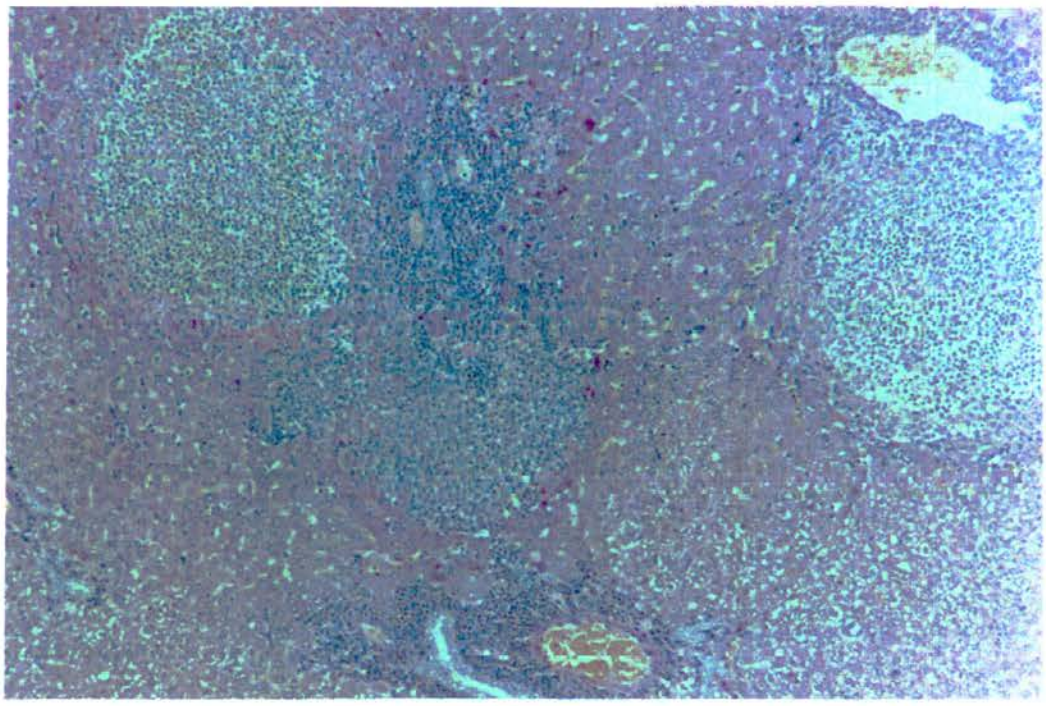


Figure 4.3a.
Transverse histological section of liver recovered at D14 from a rat infected with 20 *F. hepatica* metacercariae, stained with hematoxylin/eosin, showing multiple large necrotic foci packed with inflammatory cells.

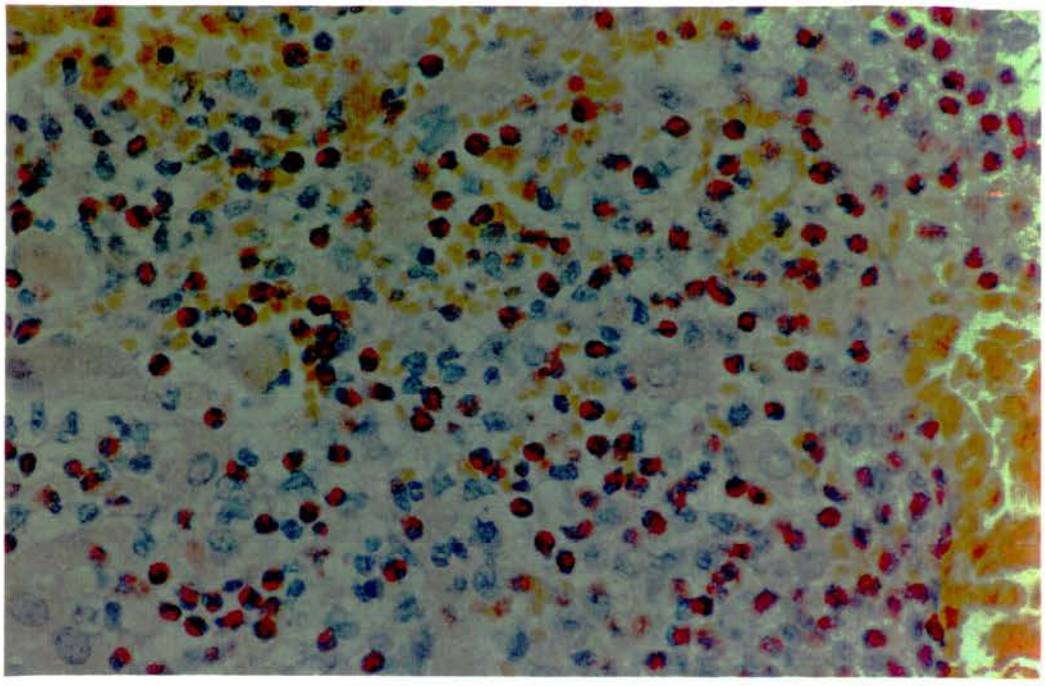


Figure 4.3b.
Transverse histological section of liver recovered from a rat infected with 20 *F. hepatica* metacercariae, stained with carbol chromatrope, showing eosinophilic infiltration.

The livers examined at D42 all showed less granulocytic reaction but there were increased interlobular fibrosis, hepatocyte degeneration and evidence of early fibroplasia. All the 13 rats that had no gross liver lesions at D56 had slight interlobular fibrosis. All the livers that had gross lesions showed varying degrees of parenchymal fibrosis, proliferation of bile ductules (Figure 4.4) and presence of pigment-laden macrophages (Figure 4.5).

4.2 PRODUCTION OF ANTISERA AGAINST *F. HEPATICA*

The mean fluke burdens from rats infected with metacercariae to produce antisera against *F. hepatica* in the three trials were 1.88 $\pm$ .273, 1.83 $\pm$ .274 and 4.29 $\pm$ .497 respectively (Figure 4.6). The mean percentage takes in the three trials were 18.1 $\pm$ 2.73, 9.3 $\pm$ 1.3 and 21 $\pm$ 2.48 respectively. The mean percentage takes in trials 1 and 3 were significantly higher than in trial 2 ($U = 474$, $P = 0.015$; $U = 241$, $P > 0.0001$) but the difference between the mean percentage takes in trials 3 and 1 was only marginally significant ($U = 481$, $P = 0.09$).

The distribution in fluke burden were similar for trials 1 and 2, at 0-7 and 0-6 respectively. Trial 3 differed in the pattern of distribution and the fluke burden ranged from 1-14 (Figure 4.7 and Appendix 1, Table A4.2).

The percent mortalities in the three trials were 7.5, 5 and 12.5 respectively.

Varying degrees of liver pathology as assessed by visual observation occurred in the infected rats (Figure 4.8). The extent and severity of observed pathology varied in each trial. Rats in trial 1 had less liver pathology compared to rats in the two subsequent trials. Of the rats that had flukes in this trial, 67% had slight liver lesions while 33% had moderate lesions. In trial 2, 13% of rats with flukes had no apparent liver lesion, while 26, 39 and 22% respectively had light, moderate and severe lesions. In trial 3, 6% of the rats had no apparent lesion, 35, 50 and 9% of the rats had light, moderate and severe lesions respectively. Pooled results from the three trials showed that in rats that had between 1 and 5 flukes, the

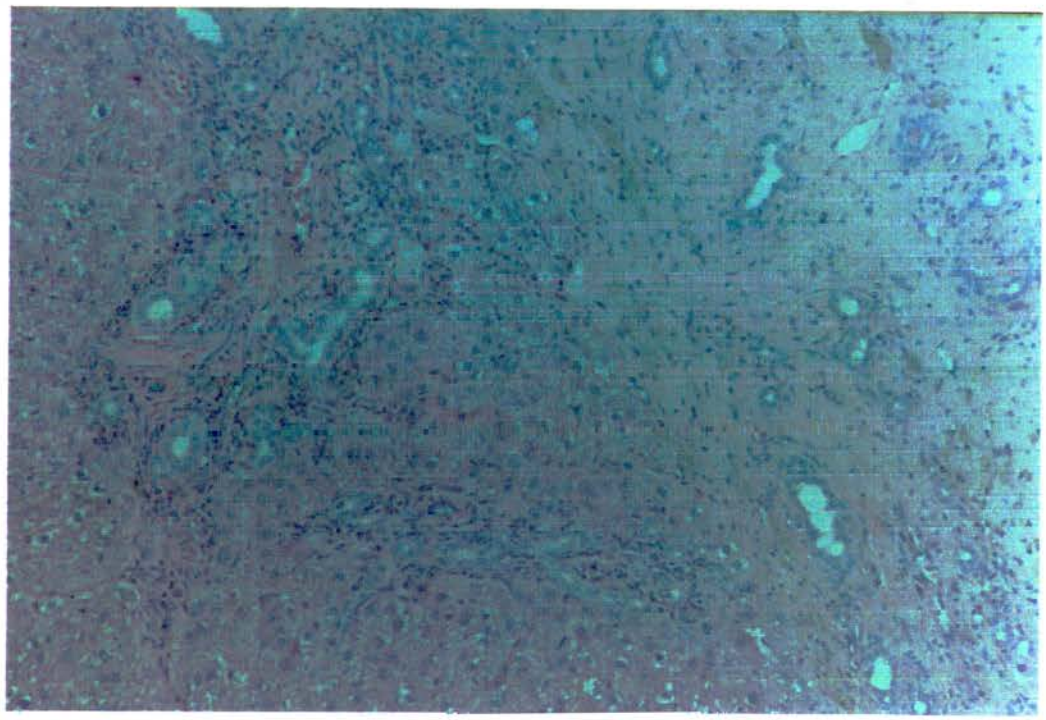


Figure 4.4.
Transverse histological section of a liver recovered at D56 from a rat infected with 20 *F. hepatica* metacercariae, stained with haematoxylin/eosin, showing bile duct proliferation and parenchymal fibrosis.

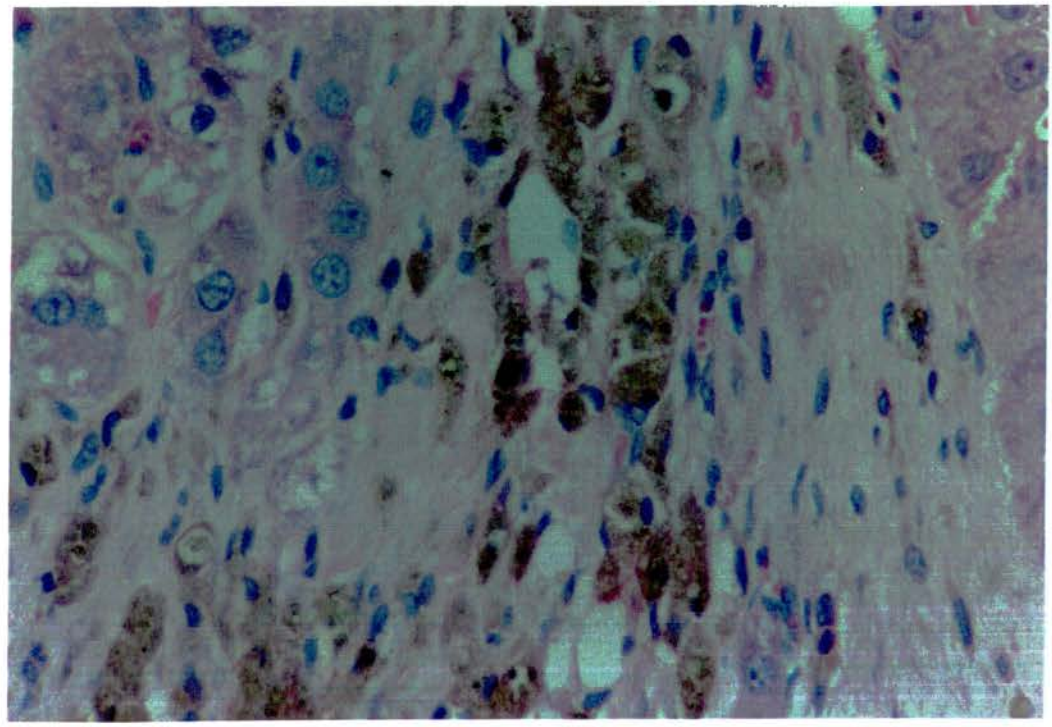


Figure 4.5.
Transverse histological section of liver recovered at D56 from a rat infected with 20 *F. hepatica* metacercariae, stained with haematoxylin/eosin, showing hepatic macrophages with heavy pigment deposits in the cytoplasm.

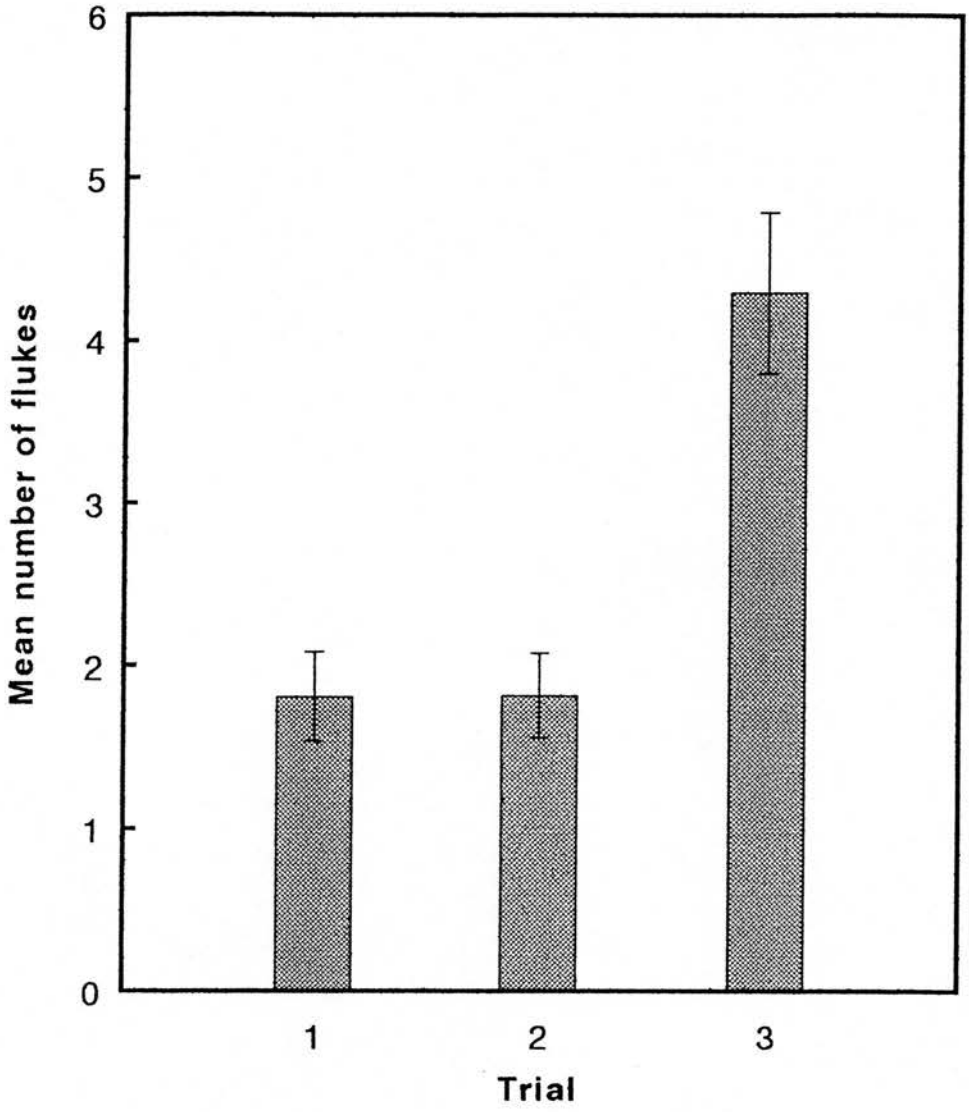


Figure 4.6
Mean (±SEM) *Fasciola hepatica* recovered recovered from rats 56 days after oral infection with 10 (trial 1) or 20 (trials 2 and 3) *F. hepatica* metacercariae.

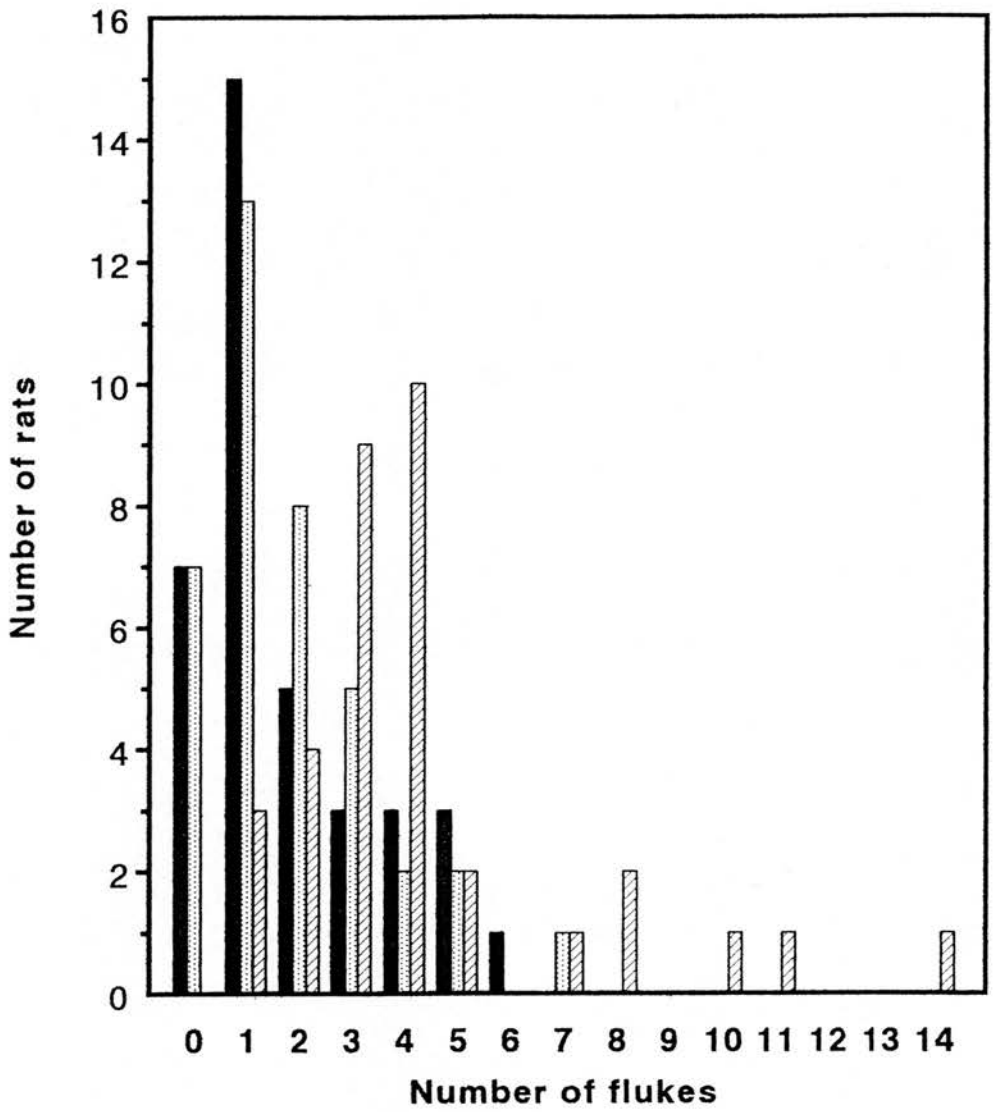


Figure 4.7
Distribution of numbers of *F. hepatica* recovered from rats 56 days after oral infection with 10 (trial 1 (■) or 20 (trials 2 (▨) and 3 (▨)) *F. hepatica* metacercariae.

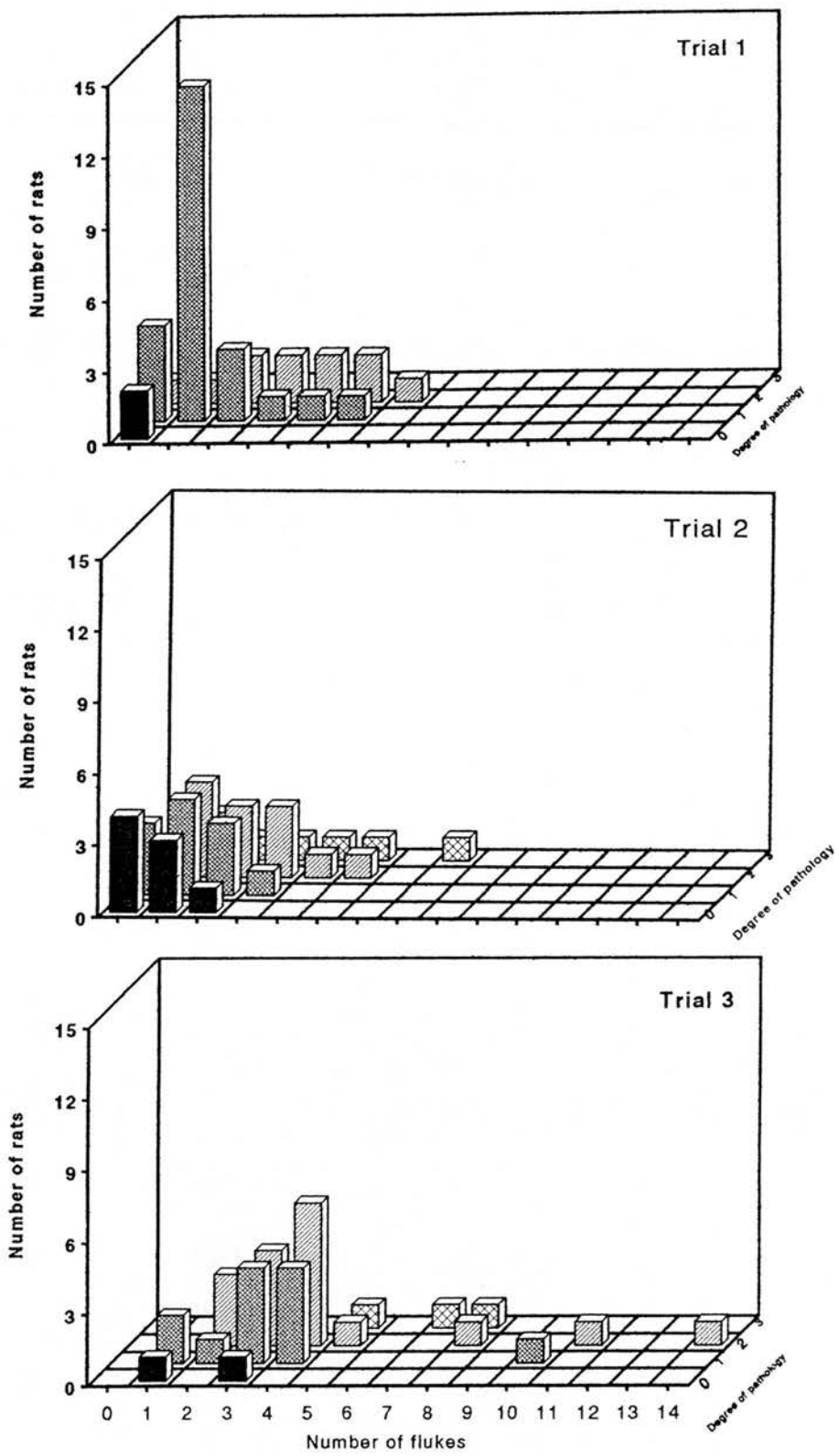


Figure 4.8.
Visually assessed liver pathology in rats infected with 10 (trial 1) or 20 (trials 2 and 3) *F. hepatica* metacercariae.

severity of liver lesion tended to increase with the number of flukes harboured. The ovcrall relationship between fluke burden and the degree of liver pathology is presented in Table 4.1. Some of the rats without flukes in trials 1 and 2 also had apparent liver pathology.

The sera obtained from the last bleeding of rats that had flukes and discernible liver pathology in the three trials were pooled and designated as I_1, I_2 and I_3 respectively. Sera from uninfected control rats were similarly pooled and designated N_1, N_2 and N_3 (Table 4.2). These sera were stored at -70°C for use in passive protection studies.

4.3 PASSIVE PROTECTION AGAINST *F. HEPATICA*

There was a higher percentage take in the first trial than in the two subsequent ones (Figures 4.9 and 4.10). The mean fluke burden of rats given antiserum in the first trial was lower than the mean for normal serum and challenge control rats. However, this was not significantly different from those of either the normal serum control ($U = 21$, $P > 0.05$) or the challenge control ($U = 18.5$, $P > 0.05$) rats. The mean recoveries from the two latter groups were not significantly different from each other ($U = 42.5$, $P > 0.05$). The same pattern of fluke burdens was observed in trials 2 and 3 but here the mean fluke burdens of rats injected with antiserum was significantly lower than that in either the normal serum control ($U = 8$, $P > 0.05$; $U = 15$, $P > 0.05$) or the challenge control groups ($U = 23$, $P > 0.05$; $U = 9$, $P > 0.05$). Once again, the mean fluke recoveries from normal rat serum and challenge control rats were not significantly different from each other in trials 2 ($U = 42$; $P > 0.05$) or 3 ($U = 37$; $P > 0.05$).

The range of fluke recovered from challenge control rats in trial 1 was similar to that of normal rat serum control rats. Either of these values was much higher than the range in rats that received rat antiserum in this trial. In trial 2, a slightly different pattern was observed. The range in challenge control rats (0-8) was

Table 4.1 Relationship between fluke burden and gross liver pathology

Number of flukes	Number of rats pathology	Assessed liver 0	1	2	3
0	14	6	7	1	0
1	31	4	20	5	2
2	17	1	7	8	1
3	16	1	5	9	1
4	15	0	5	9	1
5	7	0	1	4	2
6	1	-	-	1	-
7	2	-	-	-	2
8	2	-	-	1	1
10	1	-	1	-	-
11	1	-	-	1	-
14	1	-	-	1	-

Table 4.2 Derivation of the various sera obtained in trials 1, 2 and 3

Trial	No of rats: Fluke and pathology	No of rats: Control	Serum volume (ml)	Serum code
1	30		225	I_1
		40	300	N_1
2	31		248	I_2
		40	292	N_2
3	34		252	I_3
		40	300	N_3

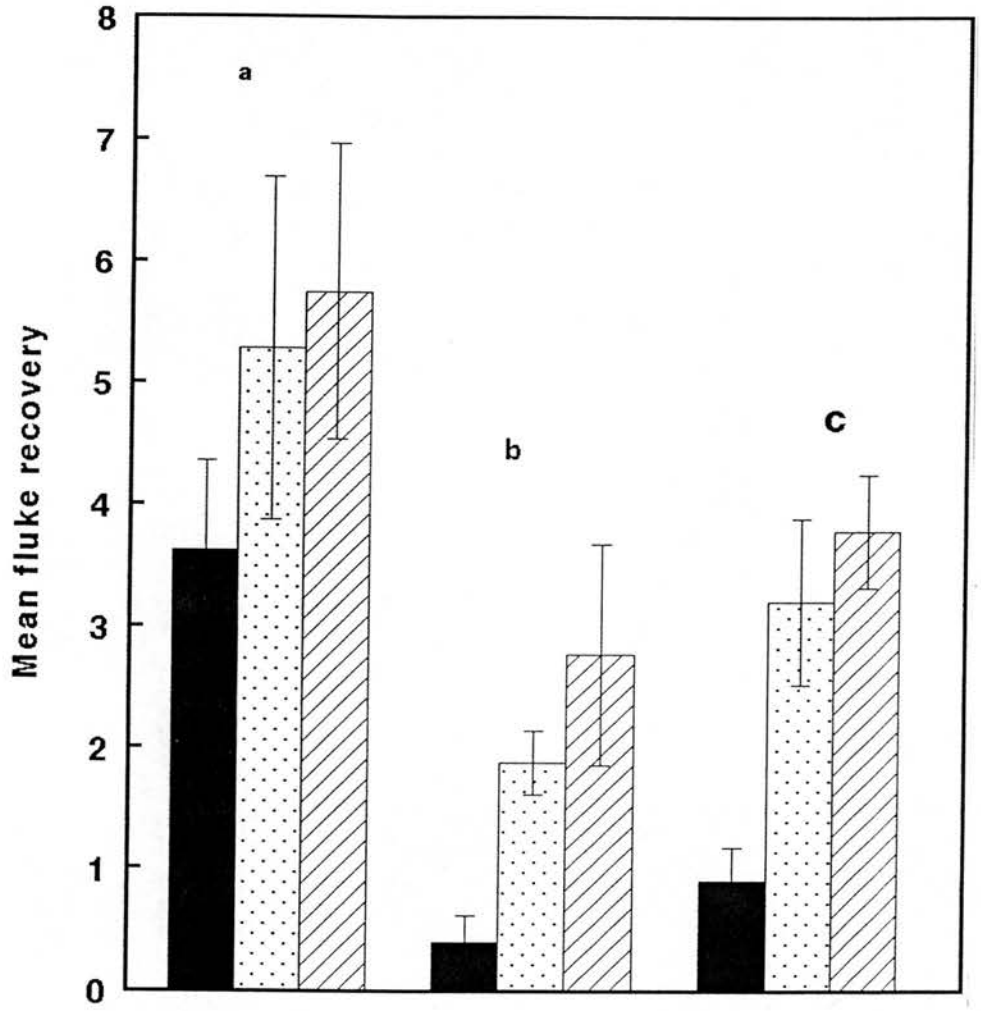


Figure 4.9

Mean (±SEM) fluke burdens recovered from rats given antiserum (▪), normal rat serum (⧈), and challenge control (▨) rats in passive protection trials 1(a), 2(b) and (c).

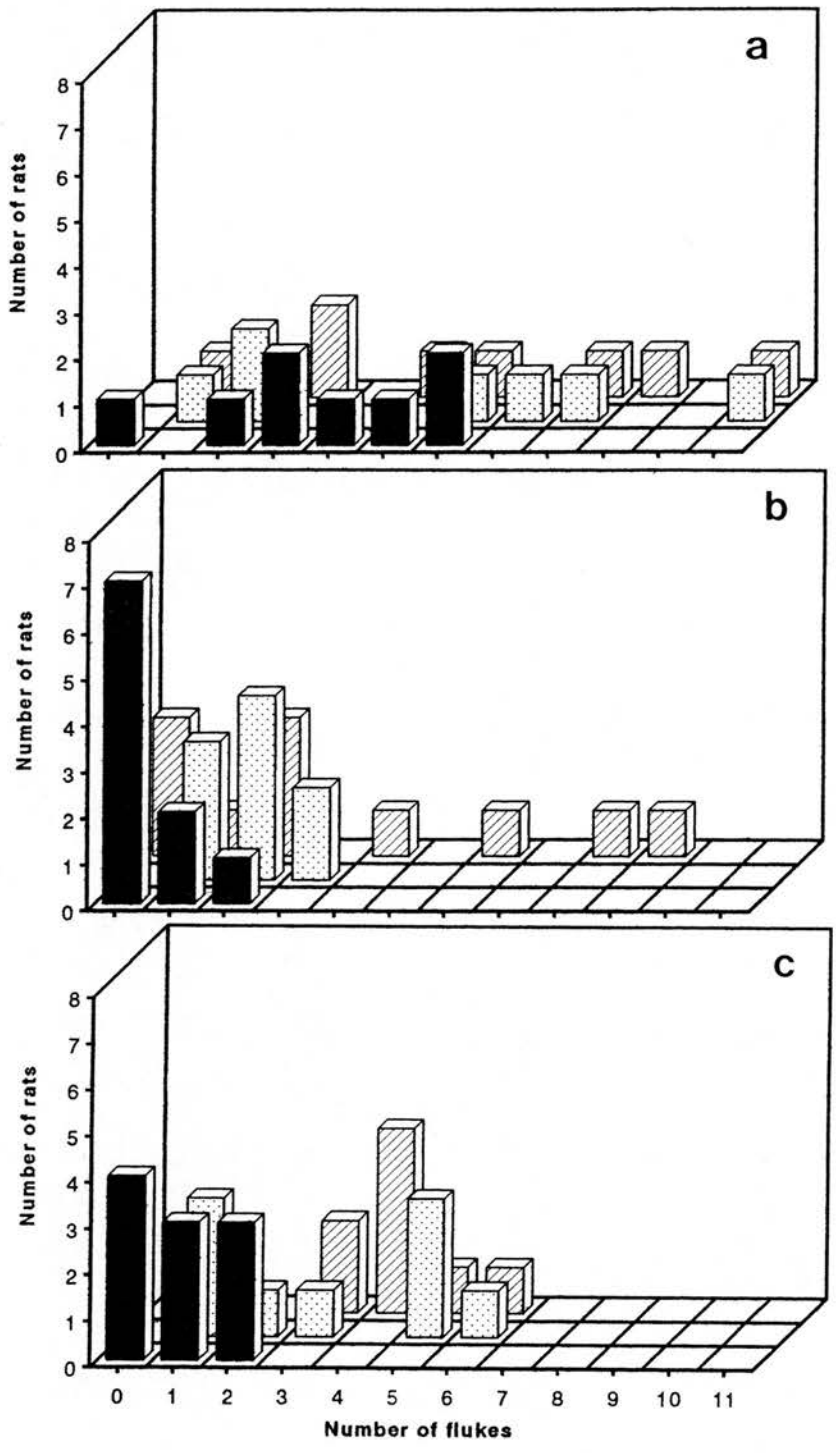


Figure 4.10.
Fluke burdens of rats given antiserum (■), normal rat serum ((▨), and challenge control rats (▨) in passive protection trials 1(a), 2 (b) and 3 (c).

much higher than that of normal serum control rats. The range in this latter group was similar to that in rats that received antiserum. The range in rats used for trial 3 was similar to that of trial 1. Thus, in the three trials the range of flukes recovered from rats that received rat antiserum were generally smaller than those of the two other groups of rats. The distribution of flukes in normal rat serum and challenge infection control rats in trials 1 and 2 followed a pattern similar to those observed in trials 1 and 2 to produce antiserum. However, this pattern was not observed in these groups of rats in passive protection trial 3 (Figure 4.10 and Appendix 1, Table A4.3).

Generally, rats that received antiserum at challenge had less liver pathology than those that received normal rat serum or challenge control rats. (Figures 4.11, 4.12 and 4.13). This difference was more marked in trials 2 and 3 (Figure 4.12 and 4.13) than in trial 1. Normal serum control rats in trial 2 had fewer discernable liver lesions than the challenge control rats (Figure 4.12b and c) but such a difference was not apparent in trial 3 (Figure 4.13b and c).

There were no significant differences in the mean sizes of flukes recovered from the three groups of rats in the three trials (Table 4.3 and Appendix 1, Table A4.3).

4.4 ANTIBODY RESPONSE TO INFECTION IN RATS

4.4.1 Titration of Parasite Extracts, Serum and Conjugate

Following titration of excretory/secretory and somatic extracts (in PBS-PI) of adult flukes against 1:100 dilutions of known positive and negative sera and 1:1000 dilutions of conjugate (Goat anti-mouse peroxidase) a concentration of 1 μg protein/ml was selected as the standard concentration for each of the extracts because the background reaction was lower at this concentration (Figures 4.14a and 4.15a).

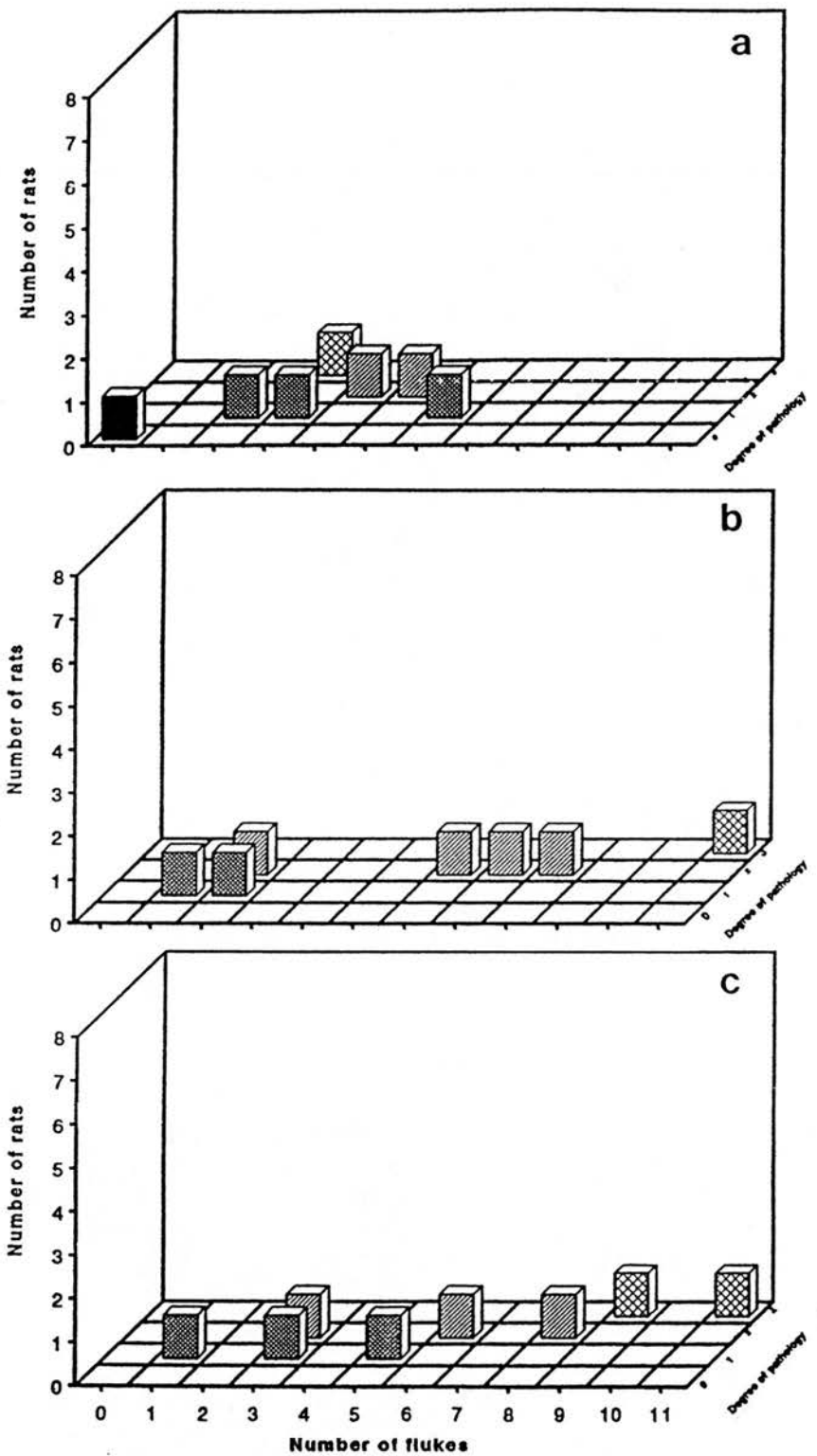


Figure 4.11.
Visually assessed liver pathology of rats given antiserum (a), normal rat serum (b) and challenge control rats (c) in passive protection trial 1.

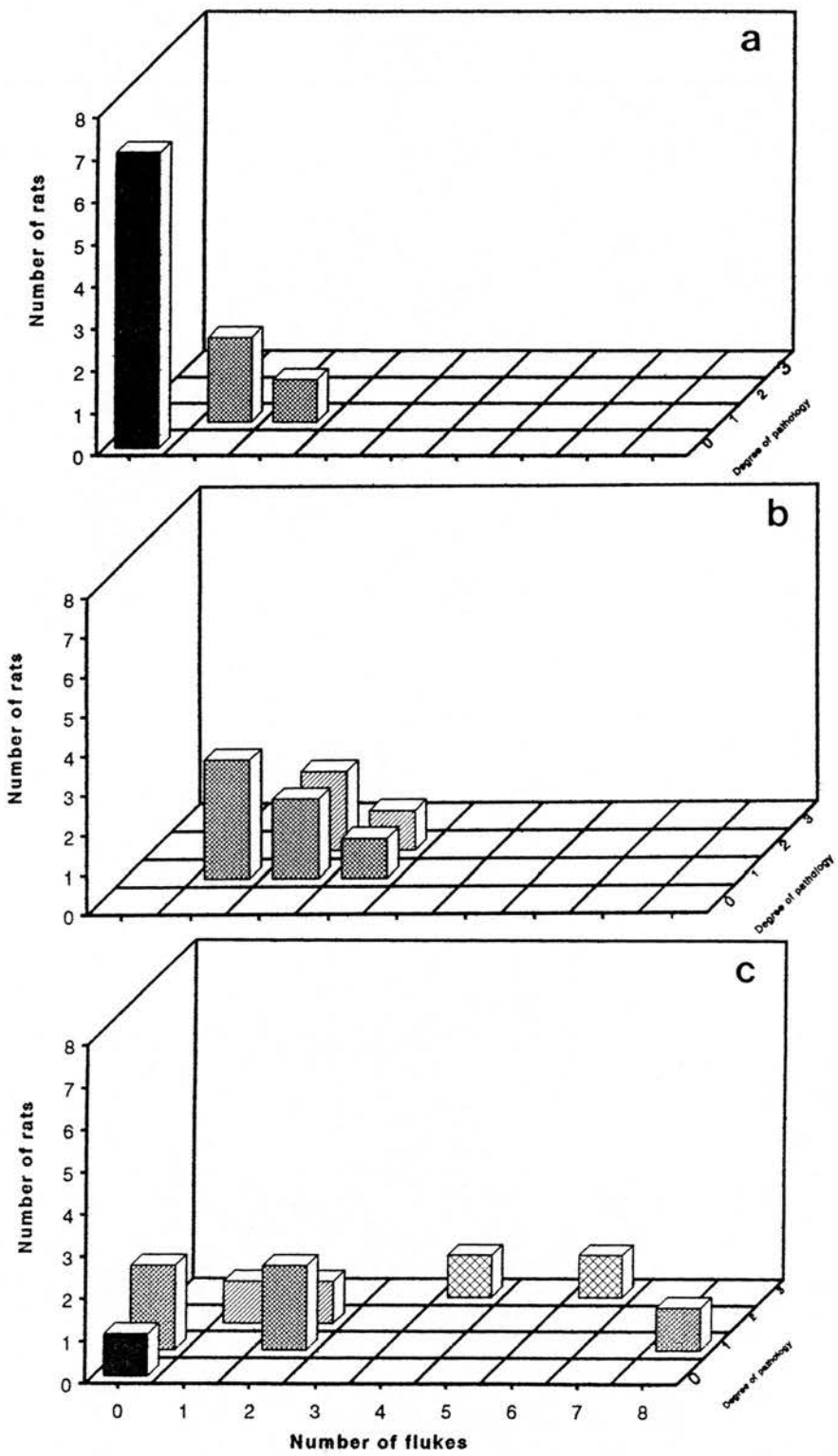


Figure 4.12.
Visually assessed liver pathology of rats given antiserum (a), normal rat serum (b) and challenge control rats (c) in passive protection trial 2.

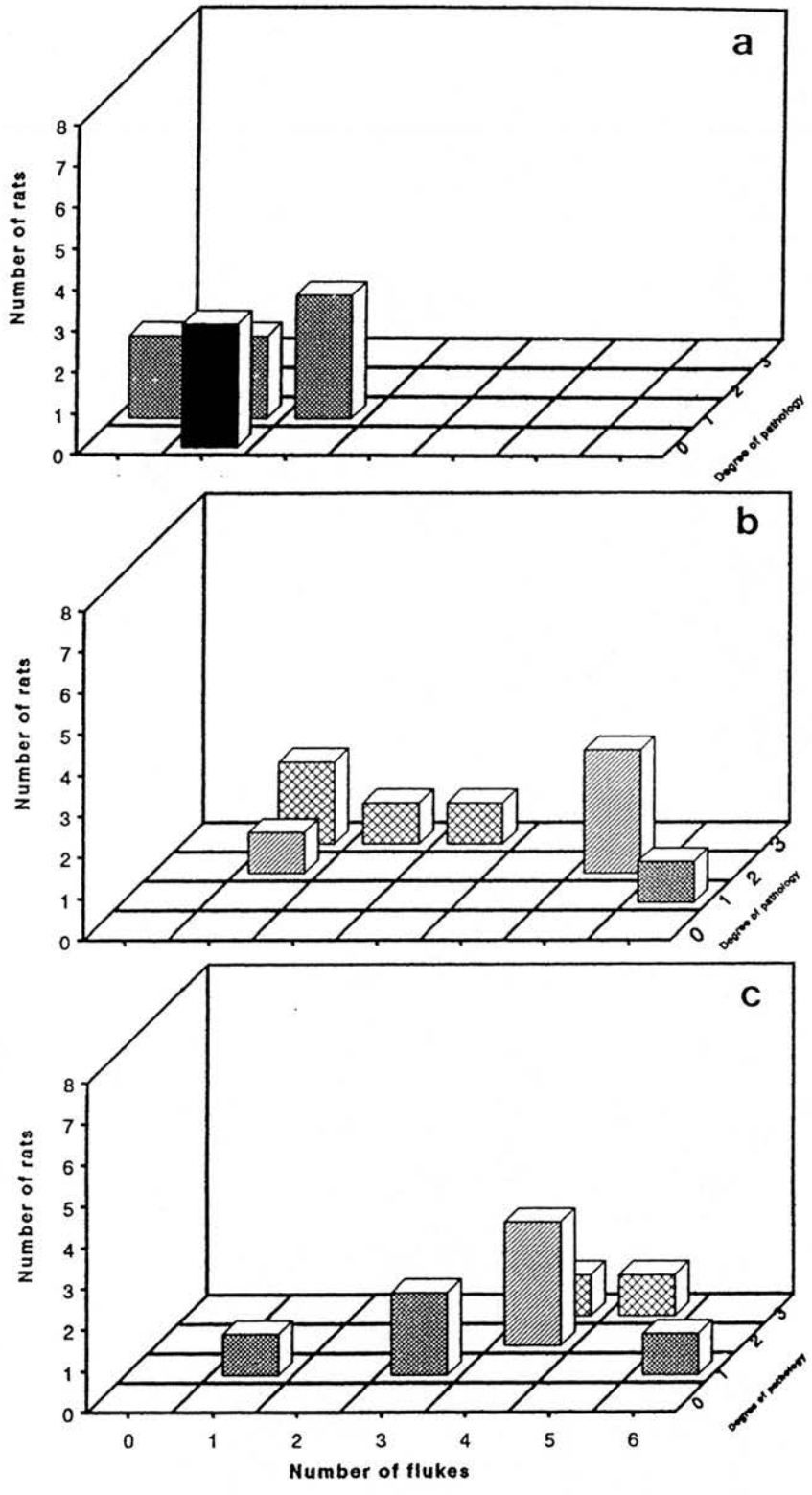


Figure 4.13.
Visually assessed liver pathology of rats given antiserum (a), normal rat serum (b) and challenge control rats (c) in passive protection trial 3.

Table 4.3 Mean measurements (mm) of flukes recovered from passive protection trials

Treatment given to rats						
+IRS		+NRS		No serum		Trial
Length	**Breadth**	**Length**	**Breadth**	**Length**	**Breadth**	
12.6±.67	6.3±.36	12.2±.76	6.47±.32	12.6±.72	6.33±.37	1
13.2±.75	6.77±41	12.47±.79	6.46±.32	12.87±.95	6.4±.49	2
15.8±1.04	7.6±.44	14.2±.73	7±.47	14.5±.98	6.7±.54	3

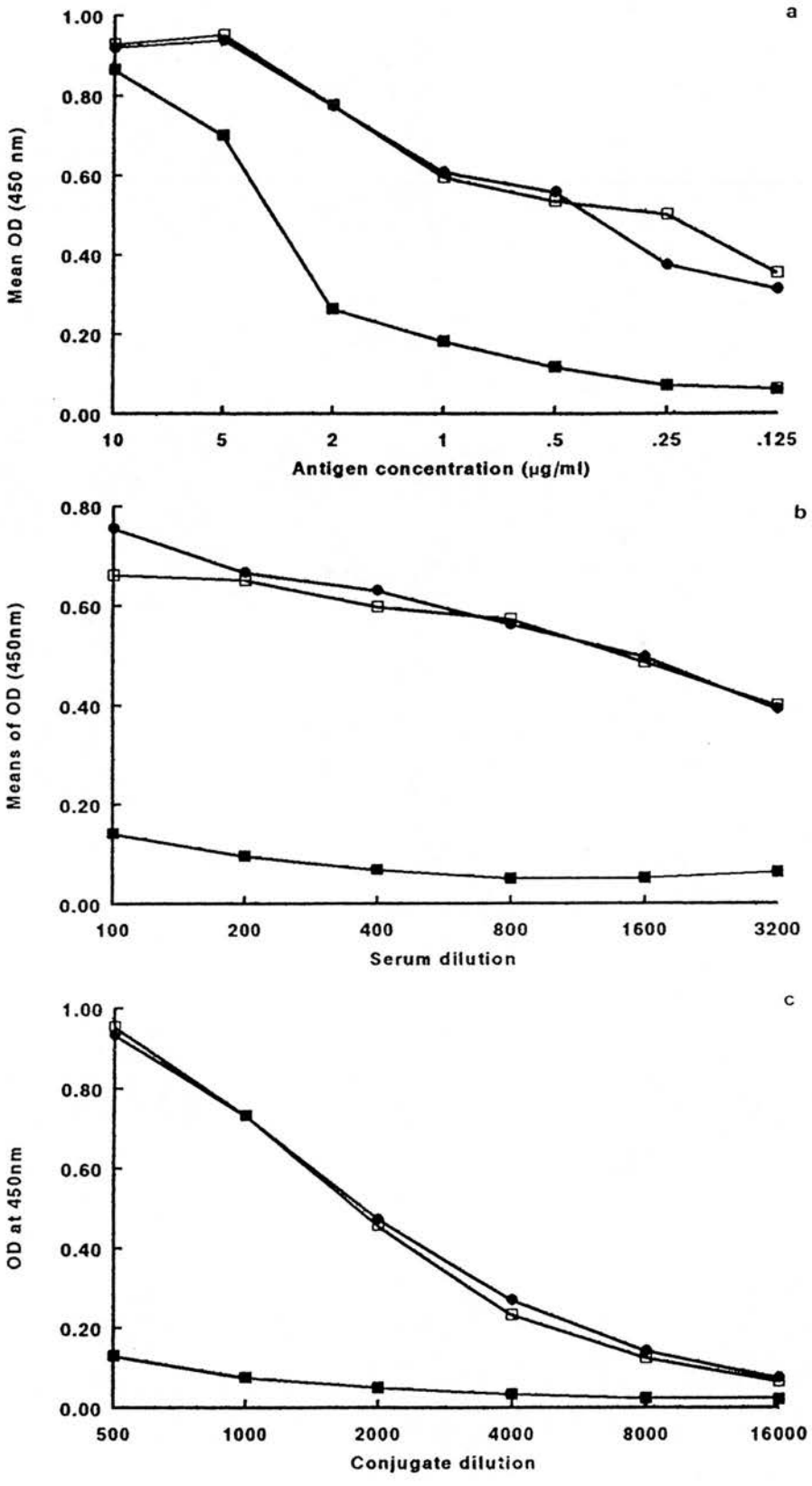


Figure 4.14.
The results of ELISA titrations performed using known positive antisera (□,●) against *F. hepatica* and known negative serum (■) to determine the optimum (a) concentration of adult ES products (b) serum dilution and (c) enzyme conjugate (goat-anti-mouse peroxidase) dilution.

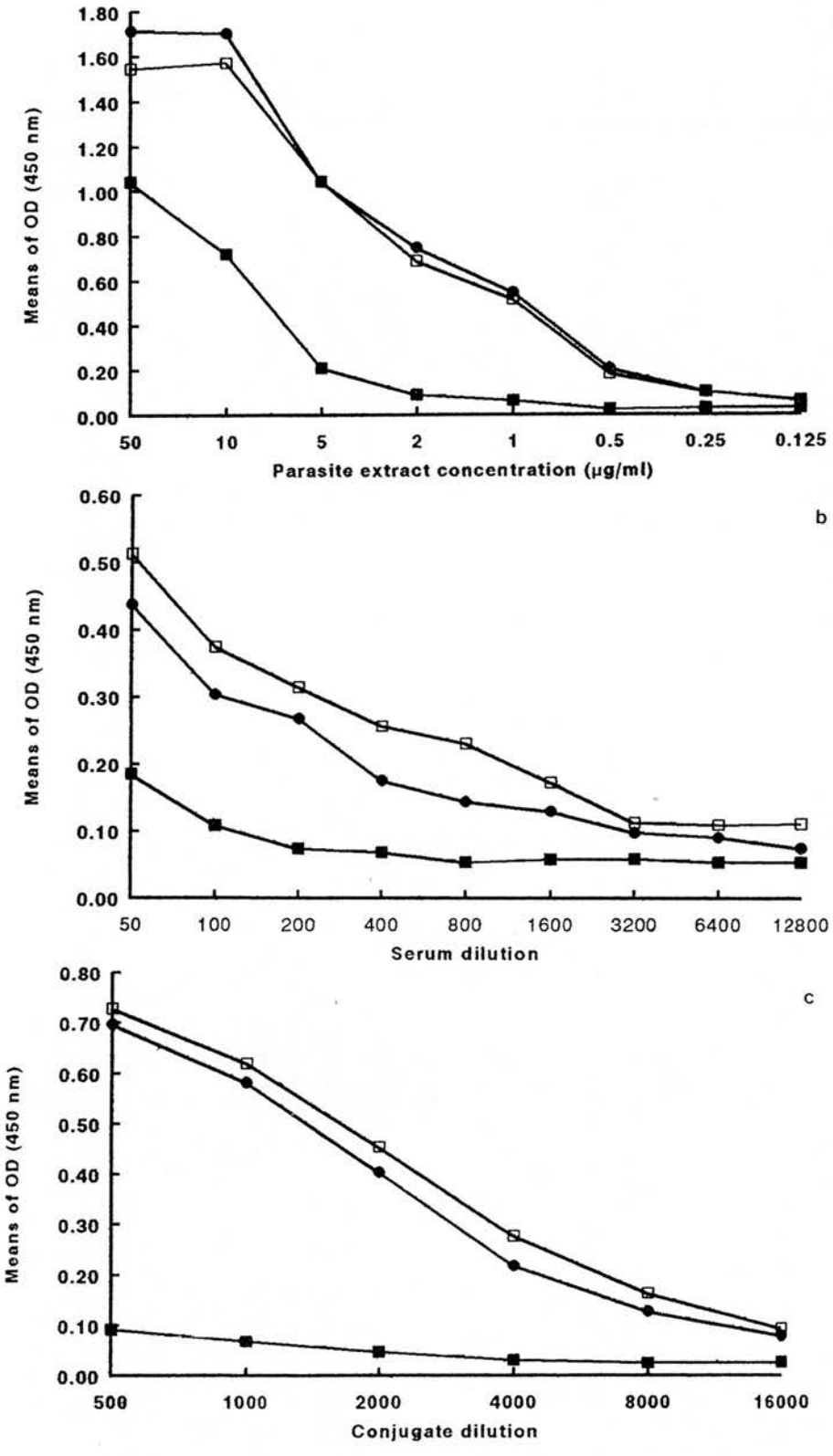


Figure 4.15.
The results of ELISA titrations performed using known positive antisera (□,●) against *F. hepatica* and known negative serum (■) to determine the optimum (a) concentration of somatic antigenic proteins (b) serum dilution and (c) enzyme conjugate (goat-anti-mouse peroxidase) dilution.

1:200. This was the optimal dilution in the titrations with ES products. The optimal serum dilution for use in ELISA with somatic fluke extract was 1:100 but this dilution was not used in order to conserve serum samples (Figures 4.14b and 4.15b).

Titration of enzyme conjugate against adult fluke excretory/secretory and somatic extracts (1 μg/ml) and 1:200 dilutions of positive and negative serum samples revealed the optimal conjugate dilution to be 1:1000 (Figures 4.14c and 4.15c).

4.4.2 Antibody Response to Primary Infection (Trials 1, 2 and 3)

Serum samples from sequential bleeds taken from the different categories of rats were screened by ELISA using ES and somatic extracts of adult fluke as antigens (Figures 4.16 and 4.17).

The response of infected rats to both parasite extracts followed similar patterns. All rats with flukes and varying degrees of liver pathology showed an antibody response within 14 days of infection. The antibody level remained high until all the trials were terminated at day 56 of infection.

Interestingly, infected rats in which no flukes were found at post mortem also showed an antibody response within 14 days of infection. In rats with no flukes but liver pathology this response peaked at day 42 of infection but persisted throughout the course of infection. In infected rats without liver pathology the antibody response plateaued after 14 or 21 days of infection.

The antibody response of uninfected control rats was negative when monitored at both the beginning and the end of trial. All the OD values for these ELISA studies are presented in Appendix 1.

4.4.3 Antibody Response of Rats used in Passive Protection Trials

The antibodies detected in the sera of rats used in the three passive protection trials to ES and somatic proteins of adult are shown in Figures 4.18 and 4.19.

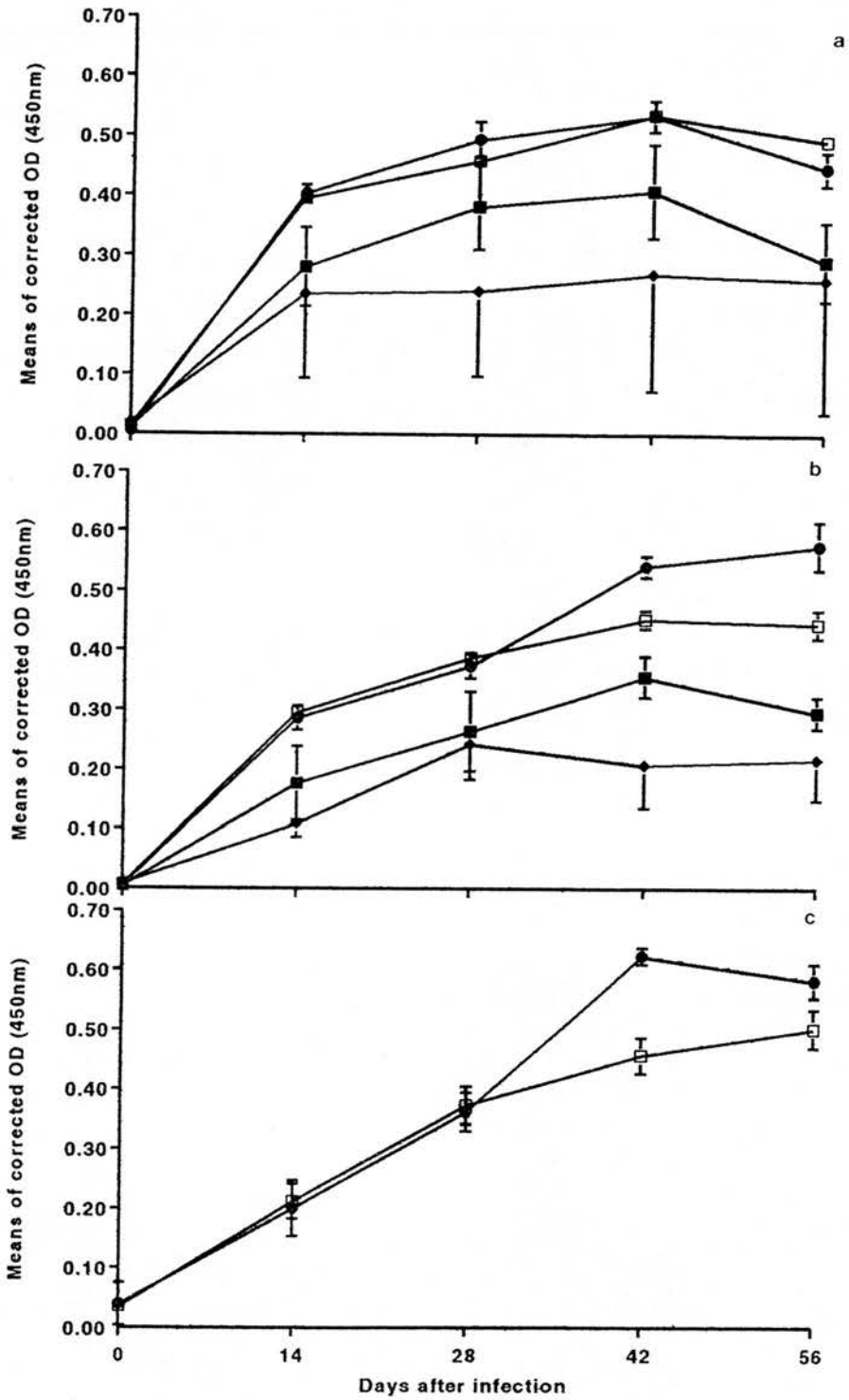


Figure 4.16.
The antibody response of rats that had 1-3 flukes (□), >4 flukes (●), no flukes but liver pathology (■) and neither fluke nor liver pathology (◆) at necropsy in trials 1(a), 2(b) and 3(c) to ES products of adult *F. hepatica*.

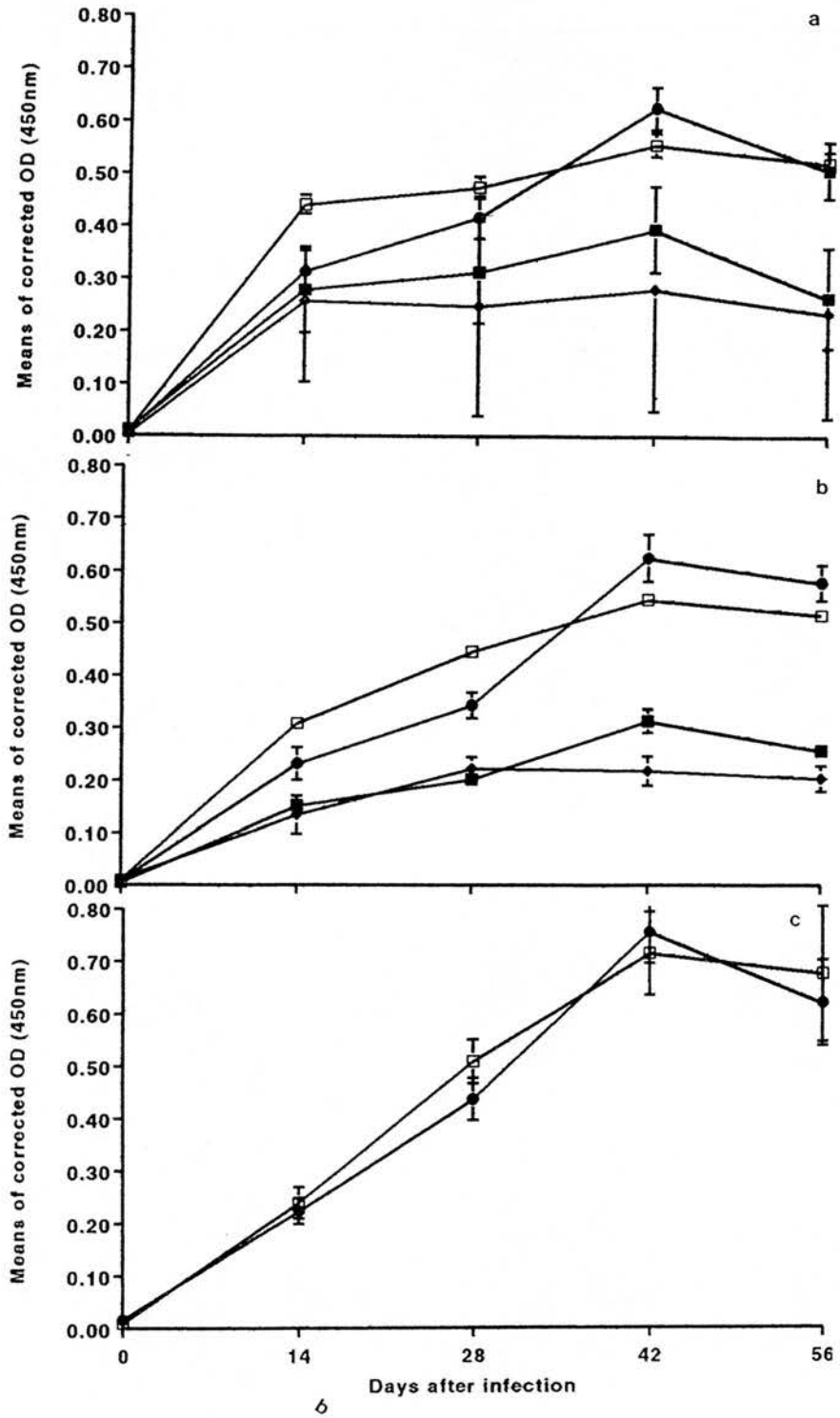


Figure 4.17.
The antibody response of rats that had 1-3 flukes (□.), >4 flukes (●), no flukes but liver pathology (■) and neither fluke nor liver pathology (◆) at necropsy in trials 1(a), 2(b) and 3(c) to somatic extracts of adult *F. hepatica*.

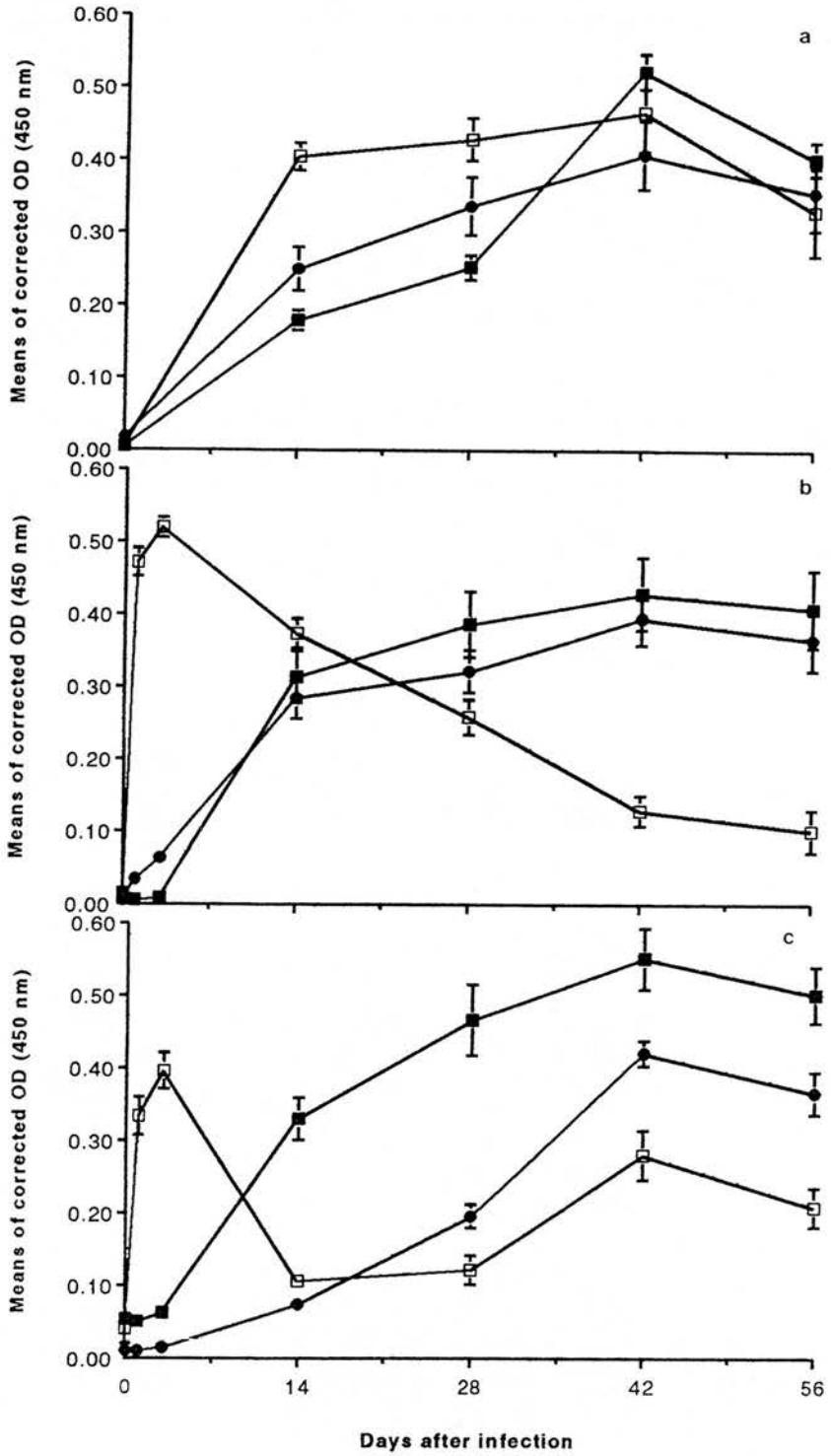


Figure 4.18.
The antibody response of rats given antiserum (□), normal rat serum (●) and challenge control (■) in passive protection trials 1(a), 2(b) and 3(c) to ES products of adult *F. hepatica*.

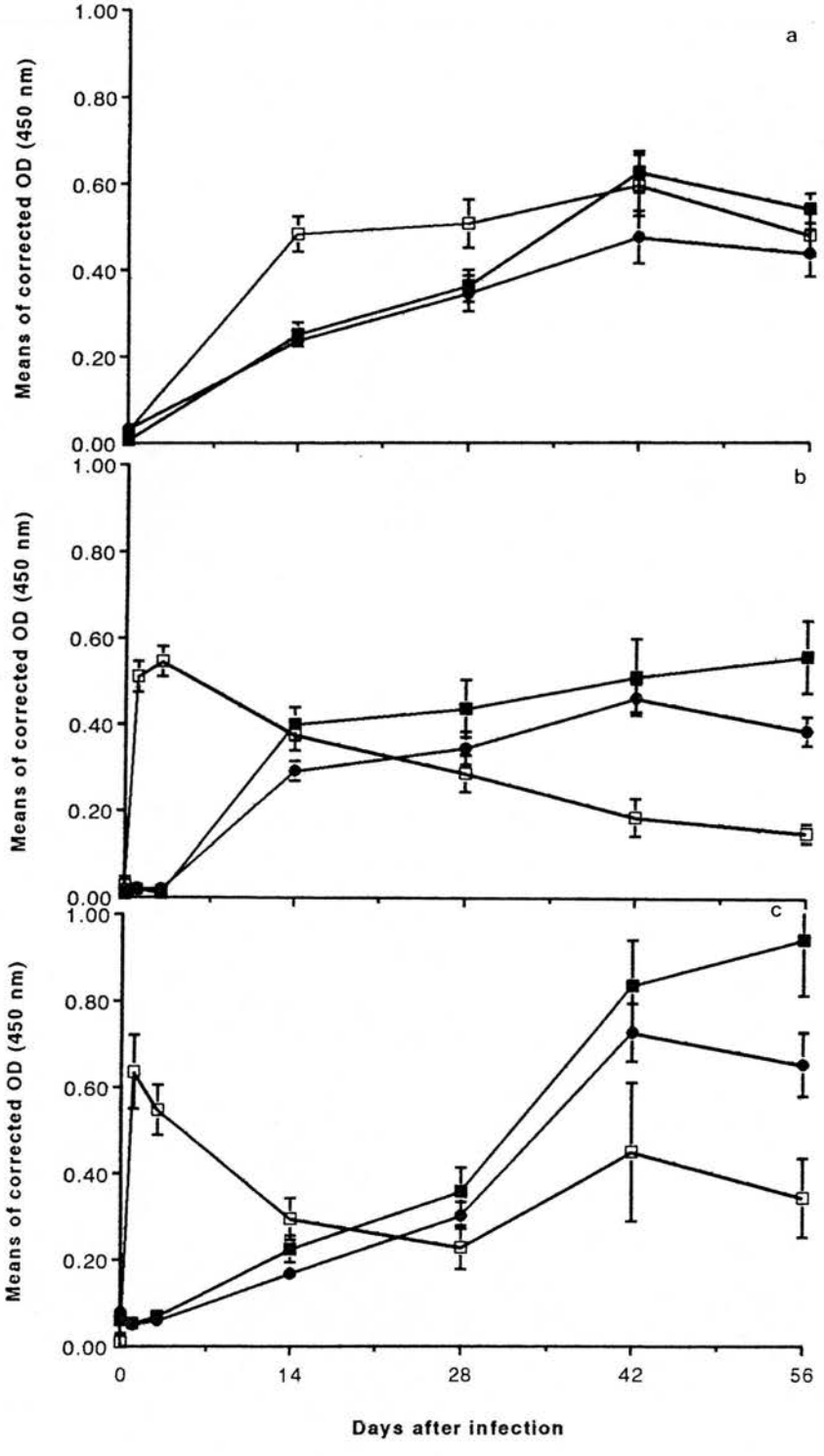


Figure 4.19.
The antibody response of rats given antiserum (□), normal rat serum (●) and challenge control (■) in passive protection trials 1(a), 2(b) and 3(c) to somatic extracts of adult *F. hepatica*.

In trial 1 antibodies were first detected by day 14 as these rats were not bled at days 1 and 3 of infection. The antibody remained high until the trial was terminated at 56 days after infection. The antibody levels of rats that received immune serum was significantly higher at days 14 (U = 0.0, P = 0.0003; U = 0.0, P = 0.0002) and 28 (U = 9, P = 0.0289; U = 3, P = 0.0011) of infection than the levels in the normal rat serum and challenge control rats.

The serum from rats that received immune serum in the second and third trials contained a very significant antibody content on days 1 (U = 0.0, P = 0.0002; U = 11, P = 0.0037) and 3 (U = 0.0, P = 0.0002; U = 10, P = 0.0029) of infection. By day 14 of infection there had been a significant (U = 4, P = 0.0006; U = 1, P = 0.0029) decline in the antibody level. In trial 2 this decline continued until the termination of the trial. In trial 3 an insignificant (U = 29.5, P = 0.131) increase was noticed by day 42 of infection. These patterns of response were markedly different from that seen in rats that received immune serum in the first trial. The normal serum and challenge control rats showed a similar pattern of response to that of similar group in the first passive protection trial.

4.5 HAEMATOLOGY

4.5.1 Changes in Packed Cell Volume

The packed cell volume of rats in the three groups did not appear to be affected by the infection. In rats that received immune serum and normal rat serum, the preinfection PCV values were significantly lower than the PCV values at the termination of the trial (U = 17.5, P = 0.015; U = 19.5, P = 0.07), but in challenge control rats these values were not significantly different from each other (U = 31.5, P = 0.45) (Figure 4.21 and Appendix 1, Table A4.5.1a)).

4.5.2 Cellular Responses to Infection

In trial 3, an increase in the percentage of circulating eosinophils was first detected by day 14 of infection in the three groups of rats. The response in the three

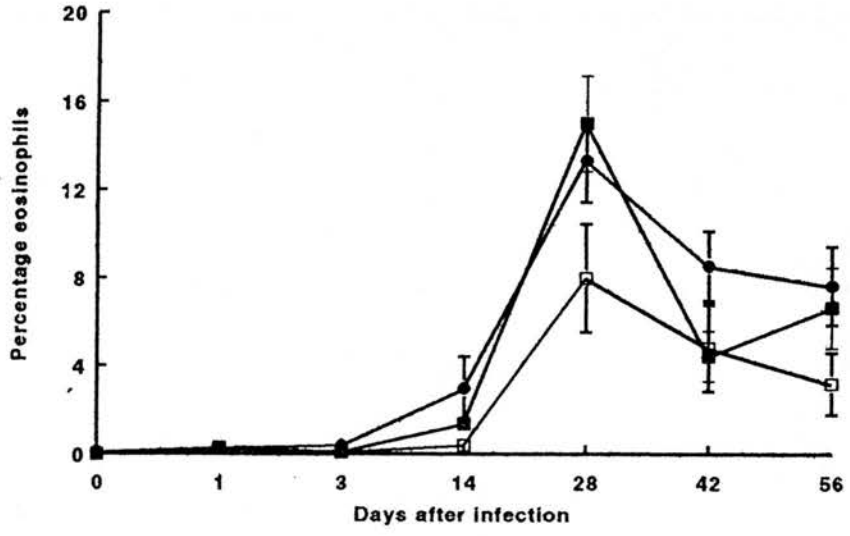


Figure 4.20.
The mean (± SEM) percentage eosinophil counts of rats that received antiserum (□), normal rat serum (●) and challenge control rats (■) in passive protection trial 3.

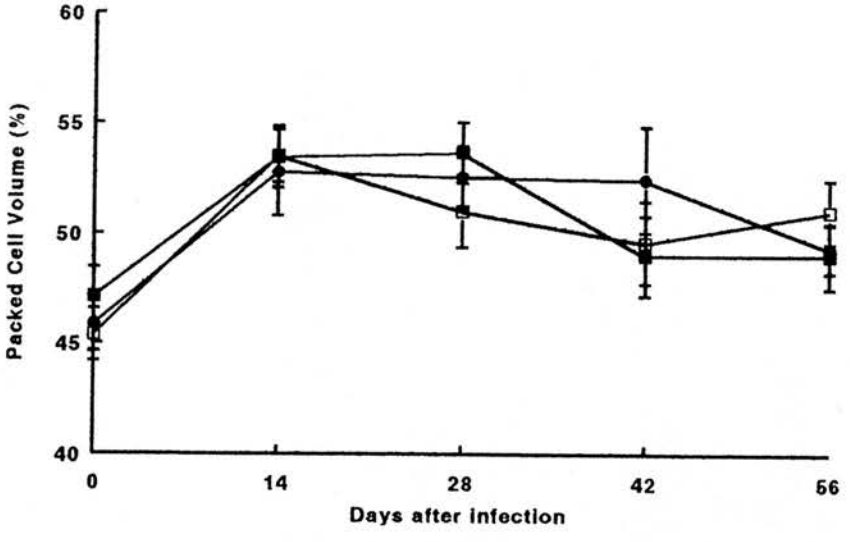


Figure 4.21.
The mean Packed Cell Volume of rats that received antiserum (□), normal rat serum (●) and challenge control rats (■) in passive protection trial 3.

groups peaked and became significant relative to their preinfection levels (U = 19.5, P = 0.0225; U = 6.5, P = 0.0031; U = 1.5, P = 0.0007) by day 28 of infection, at which time the numbers in the challenge control rats were significantly higher (U = 20, P = 0.046) than those in the rats that were injected with antiserum at challenge (Figure 4.20).

No marked changes were detected in the percentages of other leucocytes. The values are presented in Appendix 1.

4.6 WESTERN BLOTTING

4.6.1 Protein Concentration in Parasite ES Products and Somatic Preparations

The protein concentration of the ES products of D0, D1, D14 and adult flukes were 54.4, 30, 909.9 and 4000 µg/ml respectively while the protein content of PBS (somatic), N-0ctyl-glucopyranoside and cetyltrimethyl-ammonium bromide detergent surface extracts of adult flukes were 4.0, 4.55 and 0.75 mg/ml respectively.

4.6.2 Silver Staining of Parasite Excretory/Secretory Products and Extracts

Silver stained gel profiles of the proteins found in the complete culture medium, ES products of various stages of *F. hepatica* as well as in the somatic and detergent extracts of adult fluke are presented in Figures 4.22a and b.

Silver staining of the complete culture medium revealed a major protein of about 69 kDa when the gels were run under reducing or non-reducing conditions. Components analogous to this were detected in the profiles of all ages.

There were similarities but also variations in the components of ES as the flukes developed. The ES of D0 (ES_0) and D1 (ES_1) were quite similar. Both contained a major component (63-76 and 54-76) which were possibly analogous to the 69 kDa component in the complete culture medium. The four other components revealed in the ES_0 (reduced) were also present in ES_1. ES_1 had three additional

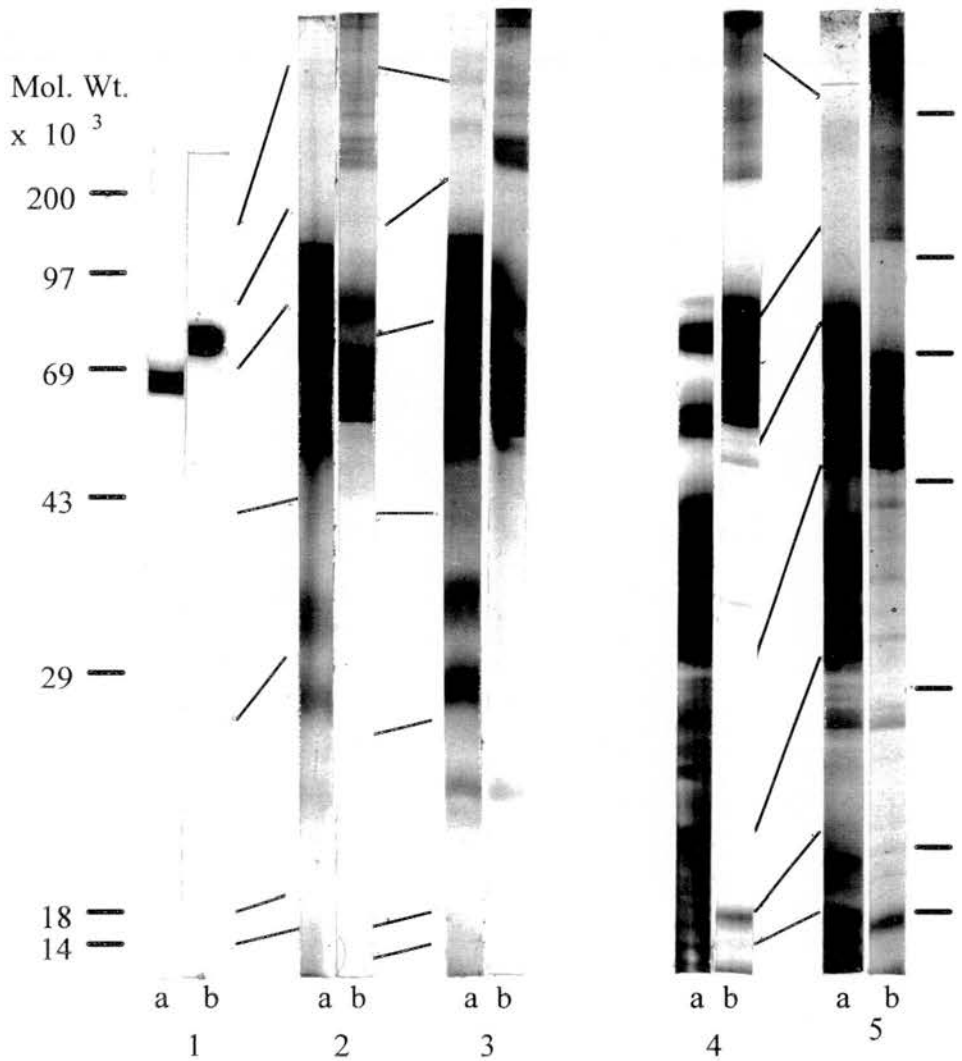


Figure 4.22a.

Silver stain of complete culture medium (track 1), and ES products of D0 (track 2), D1 (track 3), D14 (track 4) and adult (track 5) *F. hepatica* (at 15, 0.9, 1.09, 15 and 15 μg per track respectively). Gels were run under either reducing (a) or non-reducing (b) conditions.

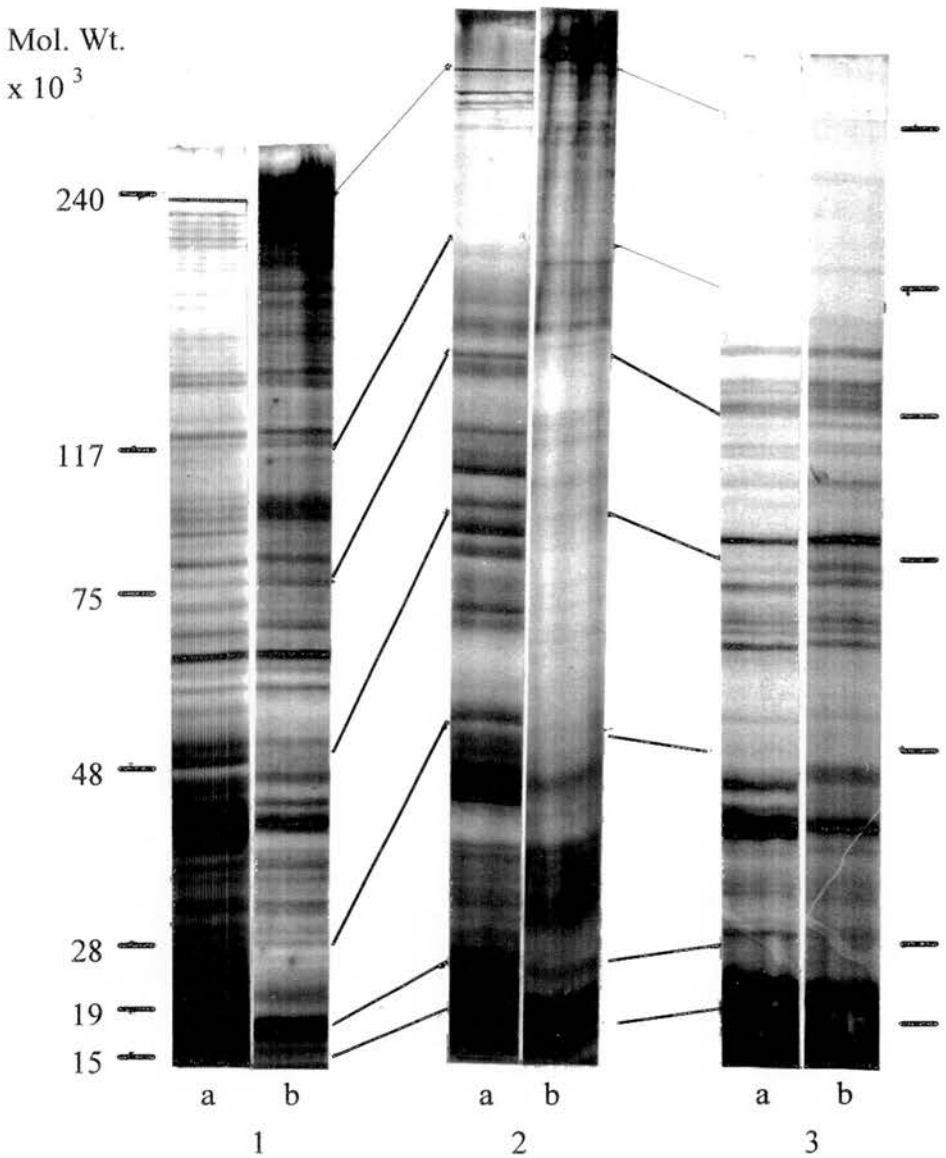


Figure 4.22b.

Silver stain of PBS-PI (track 1), NoG (track 2) and CTAB (track 3) extracts of adult *F. hepatica* (at 15μg of protein per track). Gels were run under either reducing (a) or non-reducing (b) conditions.

minor components of about 25, 180 and 200 kDa which were not detected in ES_0. Components with apparent weights of about 180 and 200 kDa which were detected in D1 ES were not present in ES_{14}. However, ES_{14} revealed new major components of about 48, 75, and 103 kDa as well minor components with molecular weights of about 15 19, 28, 42 and 124 kDa. The ES of adult flukes was quite similar to D14 ES but a few differences were noticeable. The 103 and 124 kDa components of D14 flukes were not seen in adult ES.

Changes were again observed in the profiles when the samples were run non-reduced. Analogues of the 69 kDa component in the complete culture medium were detected in the ES of all ages of flukes. ES_0 and ES_1 were again quite similar. Proteins with apparent molecular weights of 113-144 kDa was more prominent in ES_1. A component of about 26 kDa in ES_1 was not seen in ES_0. There were obvious differences between ES_1 and ES_{14}. Components with apparent weights of 176->200 kDa detected in D1 ES might possibly be analogous to the 182-222 kDa component in ES_{14}. A major component with weight of about 113-144 kDa and a minor one of about 25 kDa detected in ES_1 were both absent in ES_{14}. Conversely, three components with apparent weights of about 19, 42 and 117 kDa as well as a cluster of components with molecular weight of <15 kDa present in ES_{14} were not seen in ES_1. The non-reduced form of adult ES was again similar to that of ES_{14} but with few differences. A component with molecular weight of about 42 kDa in ES_{14} was not present in ES_A. A summary of all components detected in ES of various ages of flukes is presented in Table 4.4a anb Ь).

The somatic (PBS-PI) and NoG extracts contained more components than the CTAB extract. In the reduced state, a component of about 25 kDa was common to the three extracts although there were slight differences in the relative mobility of this component in the three extracts. A component with apparent weight of about 65-68 kDa was common to the somatic and NoG extracts. Several components that appeared quite prominent in the NoG extract were absent in the other two extracts.

Table 4.4a **Proteins identified in the silver stained complete culture medium and ES products of *F. hepatica* run under reducing conditions**

Complete medium	D0	D1	D14	D56
-	-	200	-	-
-	-	176	-	-
-	-	-	124	-
-	-	-	103	-
-	81	81	-	-
-	-	-	75	-
67	-	-	67	-
-	**63-76**	-	-	-
-	-	-	**62**	-
-	-	-	-	**60-75**
-	-	**54-76**	-	-
-	50	50	-	-
-	-	-	48	**48**
-	-	-	42	-
-	**38**	**38**	-	-
-	-	-	**36**	-
-	-	-	-	35
-	-	31	-	-
-	29	-	-	-
-	-	-	28	28
-	-	25	25	25
-	-	-	19	-
-	-	-	-	17
-	-	-	15	-
-	-	-	-	<15

Table 4.4b **Proteins identified in the silver stained complete culture medium and ES products of *F. hepatica* run under non-reducing conditions**

Complete medium	D0	D1	D14	D56
-	-	-	>240	>240
-	-	-	182-192	-
-	176-200	176-200	-	-
-	-	-	117	117-185
-	113-144	113-144	-	-
-	-	-	79-109	-
-	-	-	-	75
-	74	74-	-	-
69	-	-	72	-
-	-	-	62	-
-	56-66	56-66	-	-
-	-	-	53	-
-	-	-	-	51-65
-	-	-	-	45
-	-	-	42	-
-	-	-	-	40
-	-	-	-	36
-	-	-	-	29
-	-	25	-	-
-	-	-	19	
-	-	-	-	<15

These include components with apparent weights of about 94, 75, 62-44 and 32 kDa. In the non-reduced state, there were also similarities and variations in the profiles of the three extracts. Components with apparent weights of >240, 60 and 16-19 kDa were common to the profiles of the somatic and NoG extracts. Components with weights of about 96 and 72 kDa were also common to the profiles of the NoG and CTAB extracts. A component with molecular weight of about 18 kDa was common in the non-reduced somatic and CTAB extracts. In addition, the components of 32 and 27 kDa in the former could be the analogues of the 37 and 28 kDa components in the latter. The approximate molecular weights of the main components are presented in Table 4.5.

4.6.3 Titration of Antigens, Serum, Conjugate and Biotinylated Second Antibody

Titrations of ES products and somatic extracts of flukes against known positive and negative serum, conjugate (goat anti-rat IgG) and biotinylated second antibody (streptavidin alkaline phosphatase) using dot-blots (Bio-Rad) indicated that optimal results were achieved when the dilutions of antigen, serum, conjugate and second antibody were 3.5 μg/dot, 1:200, 1:1000 and 1:3000 respectively. These were therefore the dilutions that were used in Western blot studies (Figure 4.23).

4.6.4 Probing of Antigens

Sera collected from sequential and final bleeds of rats used in the three trials for raising antiserum as well as those from rats used for passive protection were used to probe the ES and extracts of flukes. In addition, sera collected from rats that had flukes and pathology in trial 2 was used to probe the complete culture medium.

4.6.4.1 Probing of ES of adult fluke with sera obtained from rats used to produce antiserum in trial 1 (non protective)

Serum obtained from the final bleeding of rats with fluke and liver pathology in this trial did not confer significant protection upon recepient rats. This serum as well as sera from all the sequential bleeds from these rats were used to probe ES of adult flukes. Sera similarly obtained from rats that had no flukes and

Table 4.5 **Proteins in silver-stained somatic and detergent extracts of adult *F. hepatica***

Reduced state			Non-reduced state		
Somatic	NoG	CTAB	Somatic	NoG	CTAB
-	-	-	-	>240	240
-	226-240	-	-	117	-
205-240	-	-	-	-	110
109	-	-	-	-	101
-	102	-	101	-	-
-	94	-	-	96	96
-	-	88	85	-	-
-	75	-	-	-	**72**
-	**68**	-	-	**70**	-
65-68	**65**	-	-	**60**	-
-	62	-	**58**	-	-
-	-	55	-	-	51
-	-	48	-	-	43
43	-	-	**37**	-	-
-	-	41	-	-	**32**
-	32	-	**28**	-	-
28	-	-	-	-	**27**
-	-	27	25	-	-
-	-	**26**	-	-	**18**
25	-	-	**17**	-	-
-	**22-25**	-	-	16-21	-
-	19	19	-	<15	-
-	18	18			

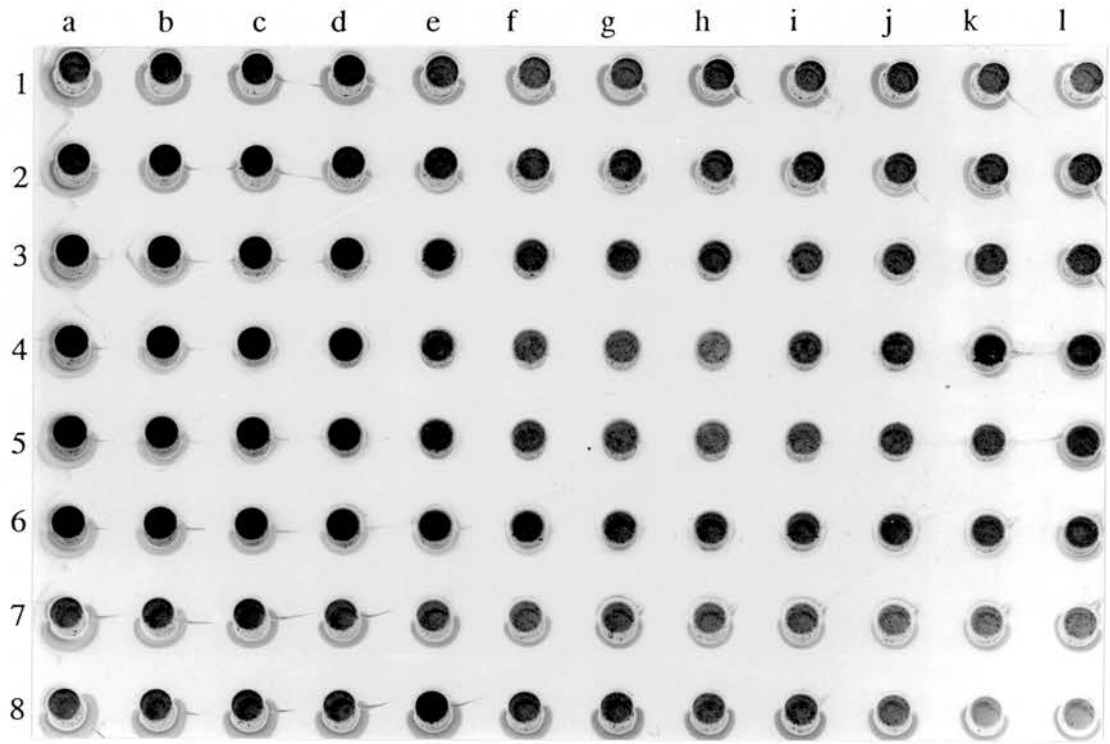


Figure 4.23.

Dot-blot titration of ES products of adult *F. hepatica* using D0 (row 2). D14 (row 3), D28 (row 4), D42 (row 5) and D56 (row 6) sera from rats with fluke and pathology (Column a - d), no flukes but pathology ((column e - g) and neither flukes nor pathology (column i - l) in trial 1.

with or without liver pathology in this trial was also used to probe the adult fluke ES.

Probing with Sera from Rats with Flukes and Pathology

D0 and D14 sera did not recognise any antigens in adult ES run under reducing conditions. D28 sera faintly recognised an antigen of about 25 kDa. This antigen was more strongly recognised by D42 and D56 sera. When used to probe the non-reduced form of ES_A, D0, D14 and D28 sera did not recognise any antigens but D42 sera again recognised an antigen of about 27 kDa. This antigen was also more strongly recognised by D56 sera. D56 sera recognised two additional antigens of about 75 and 26 kDa (Figure 4.24).

Probing With Sera From Rats With No Fluke But Pathology

D0, D14 and D28 sera recognised no antigens in the reduced form of adult ES but D42 and D56 sera recognised the 25 kDa antigen. This recognition was very weak. In the non-reduced form of adult ES, D0, D14 and D28 sera again did not recognise any antigens but D42 and D56 sera recognised the 27 kDa antigen. In addition, a doublet of about 19 kDa was recognised by both sera while an additional antigen of about 75 kDa was recognised by D56 sera (Figure 4.25).

Probing With Sera From Rats With Neither Fluke Nor Pathology

Sera obtained from sequential and final bleeds of these rats did not recognise any antigens in ES_A whether reduced or non-reduced (Figure 4.26).

4.6.4.2 Probing of various fluke extracts with sera obtained from sequential bleeds in trial 2 to produce (protective) antiserum

Excretory/Secretory Products of D0, D1, D14 and Adults

D0, D14 and other sera collected subsequently from rats that had fluke and liver pathology did not detect any antigens in complete culture medium run under reducing or non-reducing conditions.(Figure 4.27).

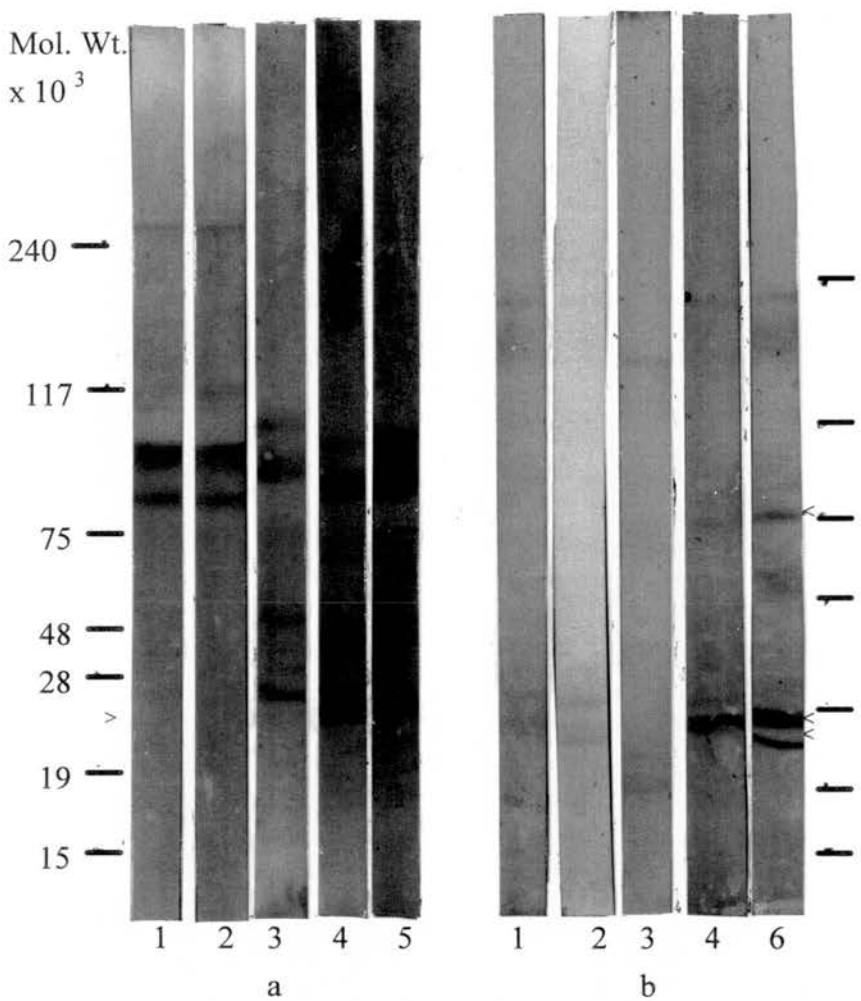


Figure 4.24.

Western blots of ES products of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and liver pathology in trial 1. Gels were run under either reducing (a) or non-reducing b) conditions.

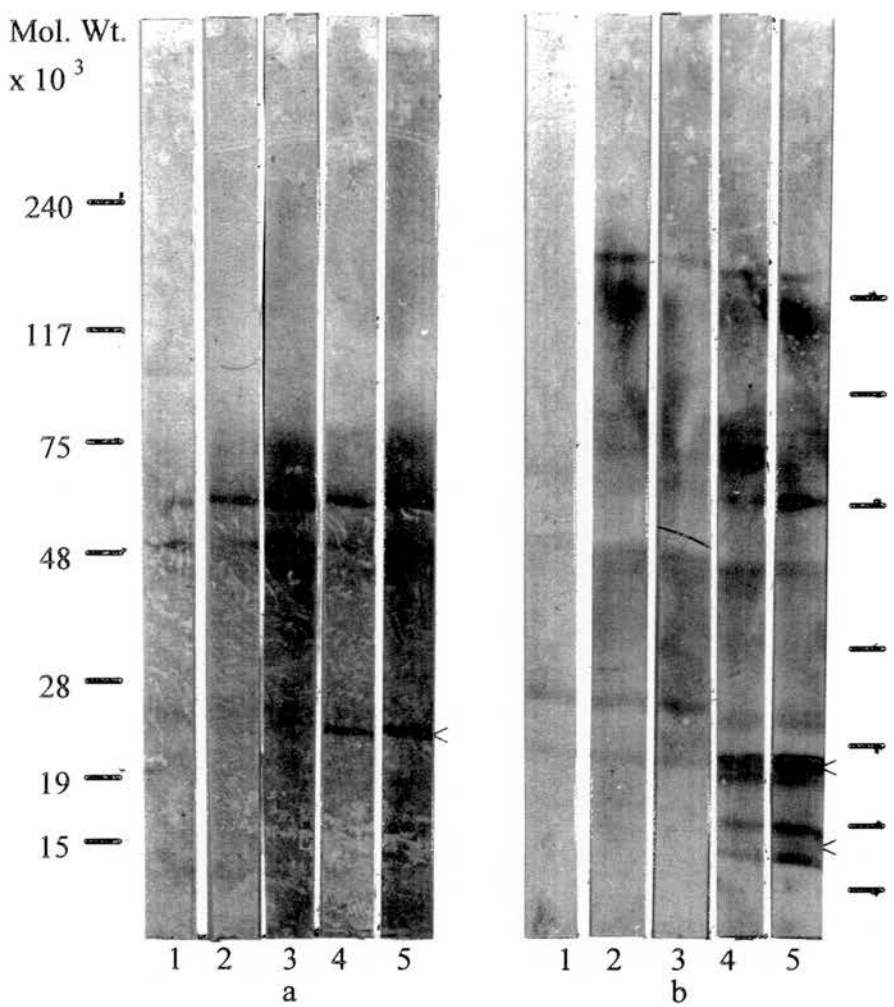


Figure 4.25.

Western blots of ES products of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with no flukes but pathology in trial 1. Gels were run under either reducing (a) or non-reducing (b) conditions.

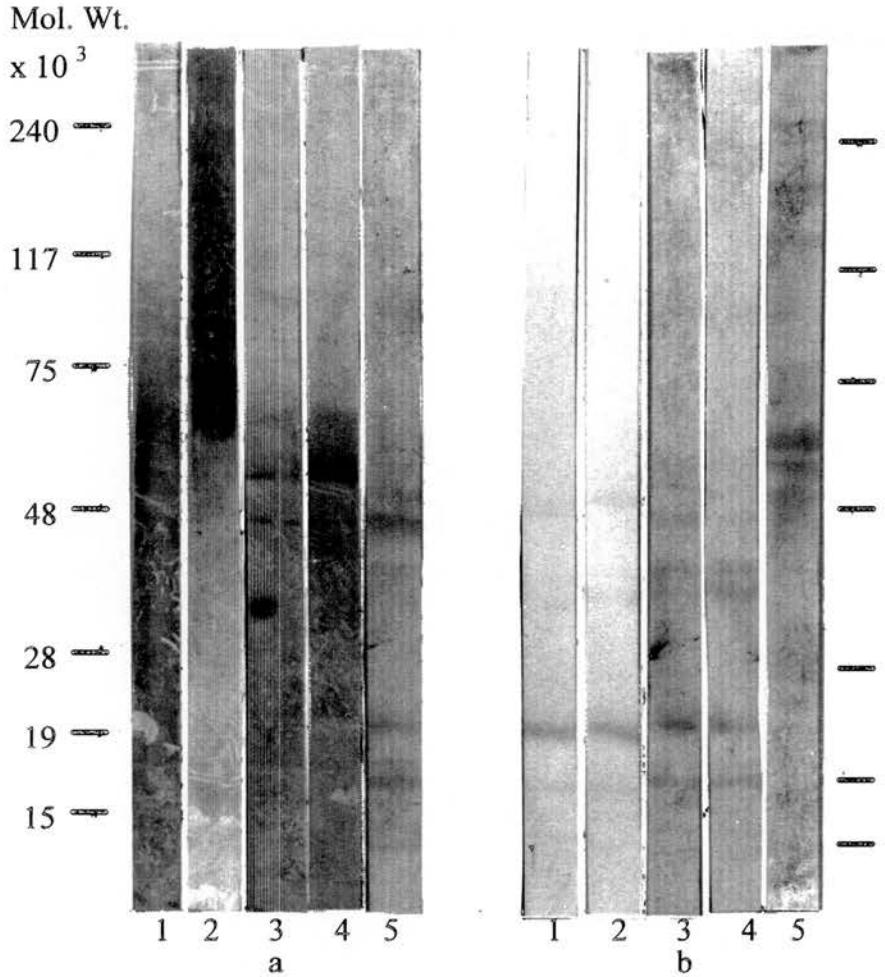


Figure 4.26.

Western blots of ES products of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with neither flukes nor pathology in trial 1. Gels were run under either reducing (a) or non-reducing (b) conditions.

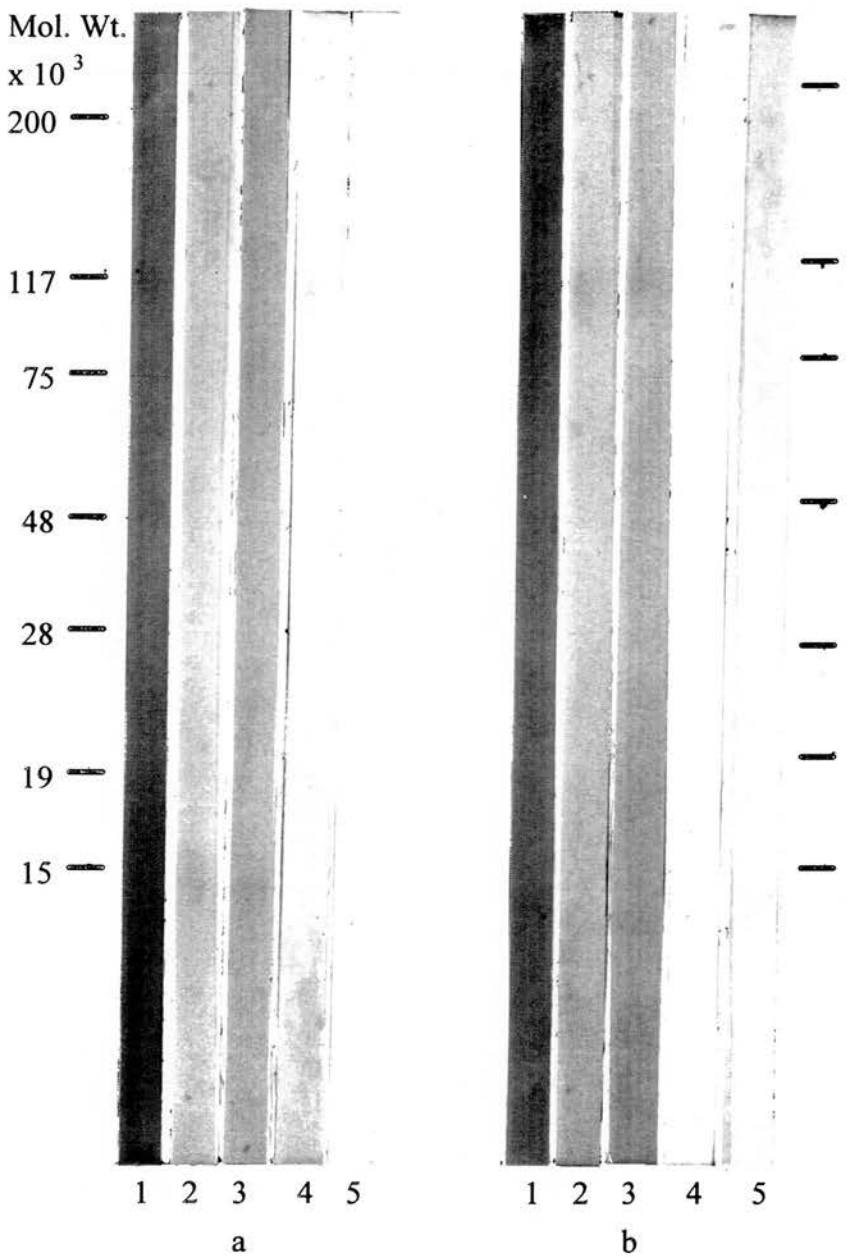


Figure 4.27.

Western blots of complete culture medium probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

D0, D14 and D28 sera from rats that had flukes and liver pathology did not recognise any antigens in the ES_0 or ES_1 run under reducing conditions. An antigen of about 191kDa was identified by D42 and D56 sera (Figures 4.28 and 4.29).

D0, D14 and D28 sera did not recognise any antigens in the ES_{14} whether gels were run under reducing or non-reducing conditions. A 25 kDa doublet was detected by D42 and D56 sera (Figure 4.30).

D0, D14 and D28 sera from rats that had flukes and pathology did not recognise any specific antigen in ES_A run under reducing or non-reducing conditions. A 25 kDa doublet was recognised in the reduced form of the ES by D42 and D56 sera. Sera collected on these days also detected a major antigen of about 26 kDa and a minor antigen of about 27 kDa in the non-reduced form of the extract (Figure 4.31).

Pooled sera obtained from sequential and final bleeds of rats that had neither fluke nor pathology in this trial did not detect any antigens in ES_A (Figure 4.32).

Somatic (PBS) Extract of Adult Fluke

D0 sera did not react with any components in somatic extract run under reducing conditions. However, D14 and D28 sera faintly recognised a 38 kDa antigen while D42 and D56 (I_2) sera recognised three additional antigens of <15, 23 and 24 kDa. When the extract was run under non-reducing conditions, again D0 sera did not react with any components while D14 and D28 sera faintly recognised a singlet of about 23 kDa and two doublets of about 43 and 48 kDa. D42 sera did not recognise the 48 kDa doublet but the 23 kDa antigen was more strongly recognised. D56 sera (I_2) recognised the same antigens that were detected by D42 sera but two additional antigens (<15 and 63 kDa) were also recognised (Figure 4.33).

Surface NoG Extract of Adult Flukes

No antigens were recognised by D0 sera but D14 and D28 sera both faintly recognised five antigens with apparent weights of <15, 25, 38, 48 and 62 kDa in this extract run under reducing conditions. D42 sera detected these antigens but the

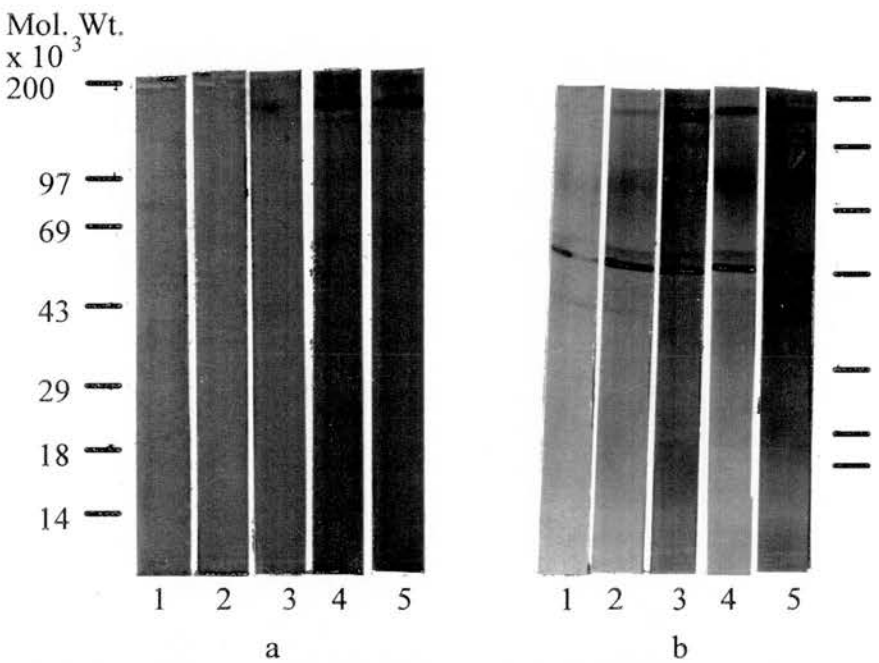


Figure 4.28.

Western blots of ES products of D0 *F. hepatica* collected over 0-24h (a) and 24-48h (b) and probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gels were run under reducing conditions

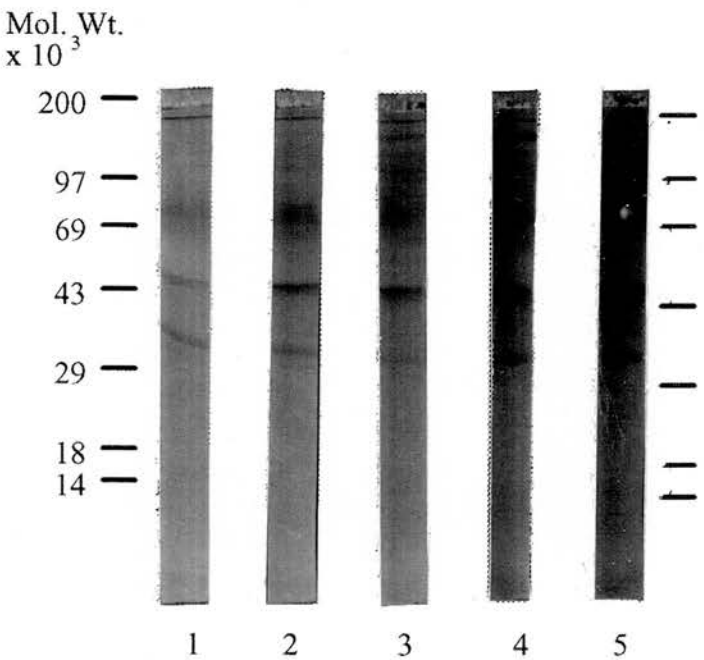


Figure 4.29.

Western blots of ES products of D1 *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gel was run under reducing conditions

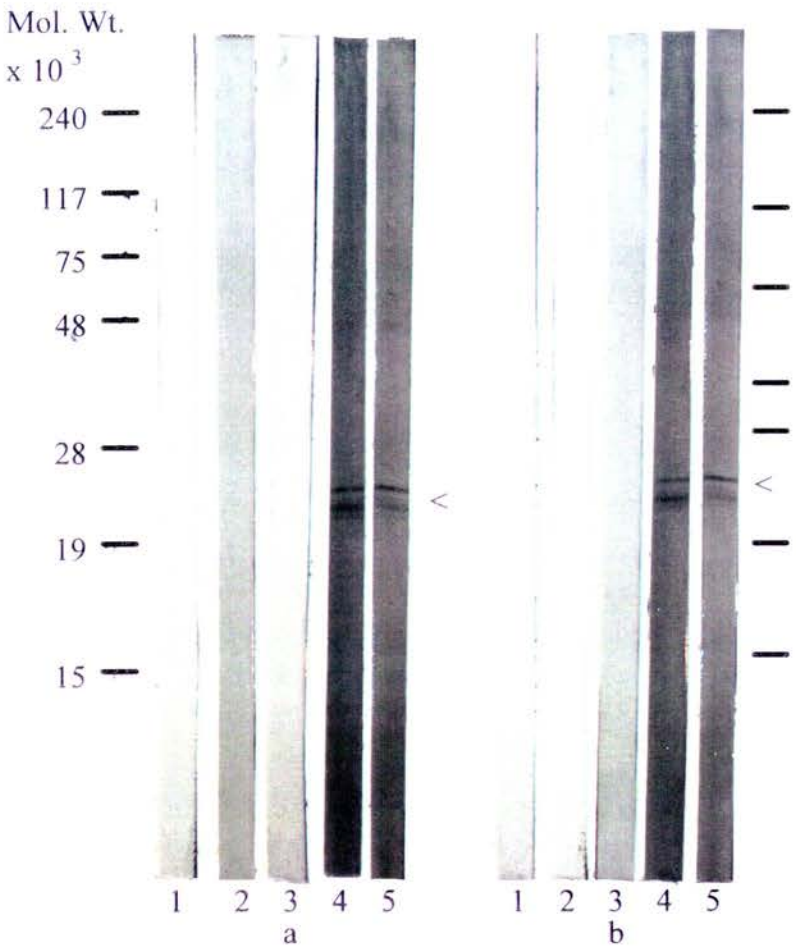


Figure 4.30.

Western blots of ES of D14 *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

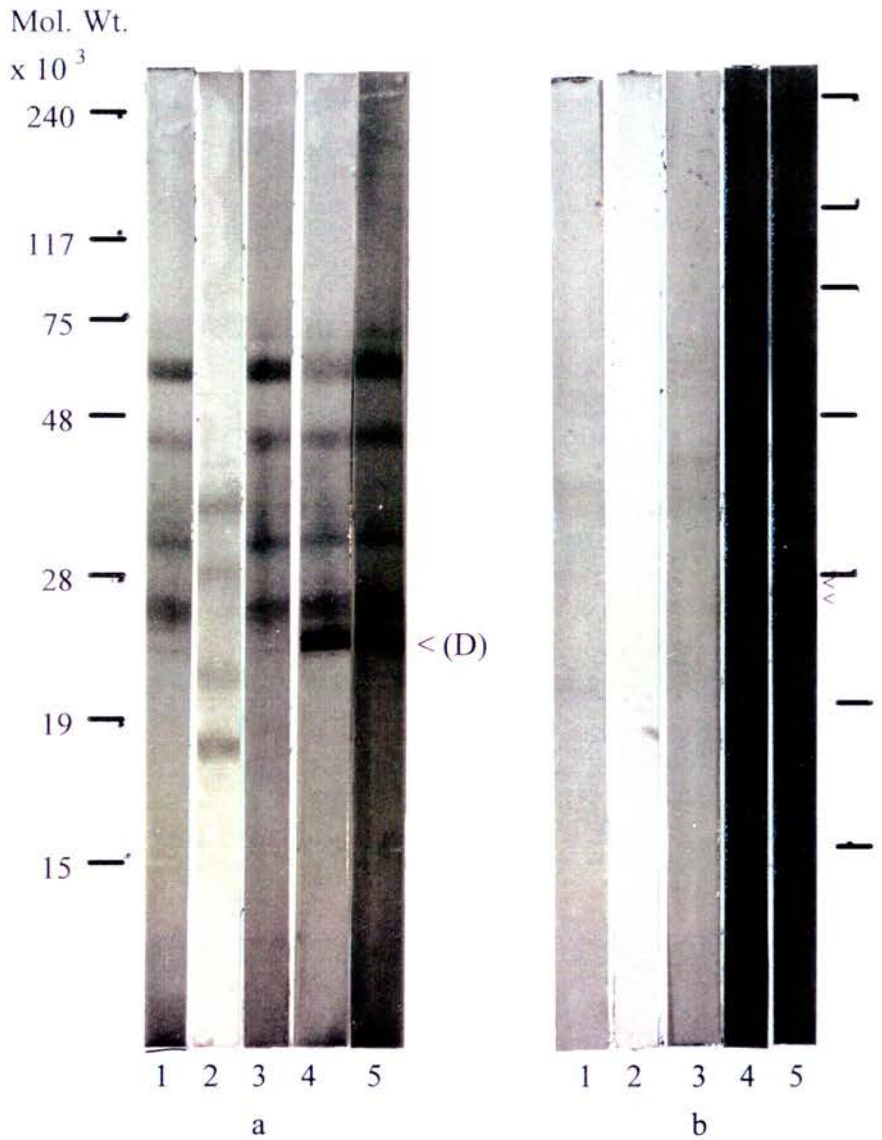


Figure 4.31.

Western blots of ES products of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

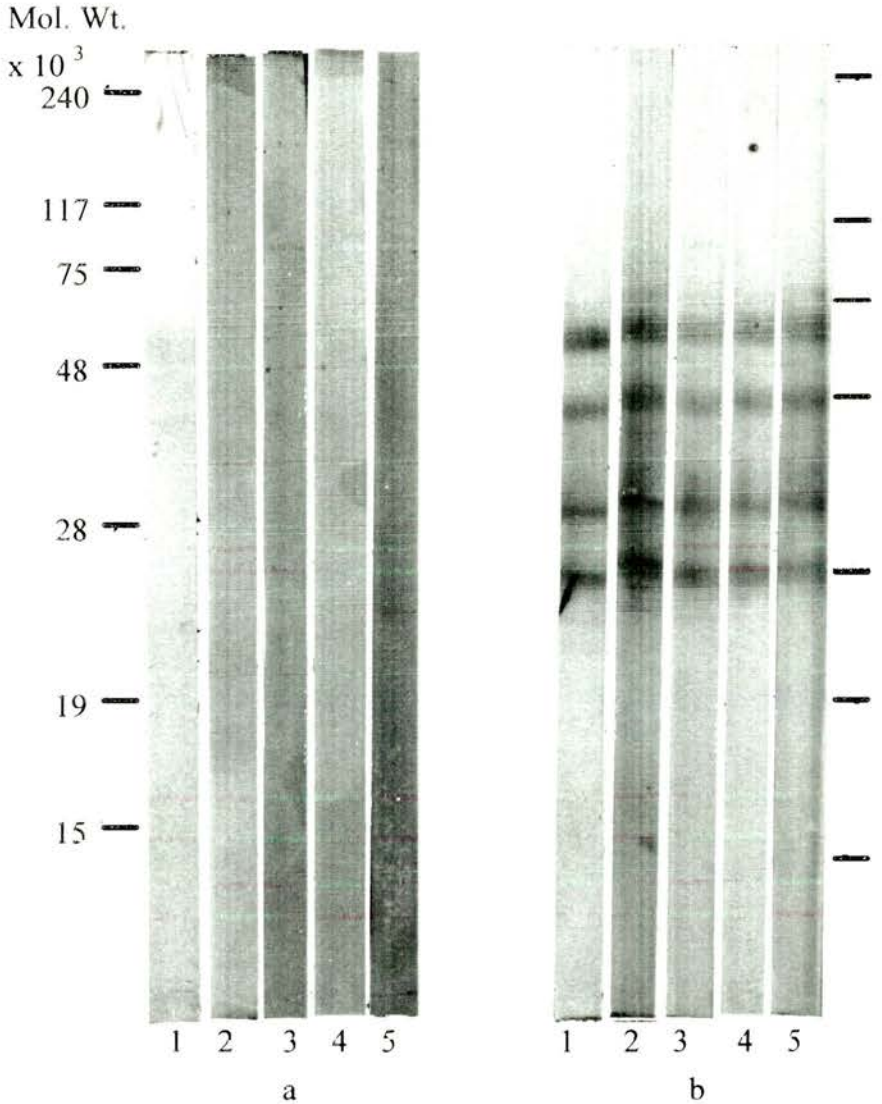


Figure 4.32.

Western blots of ES products of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with neither flukes nor pathology in trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

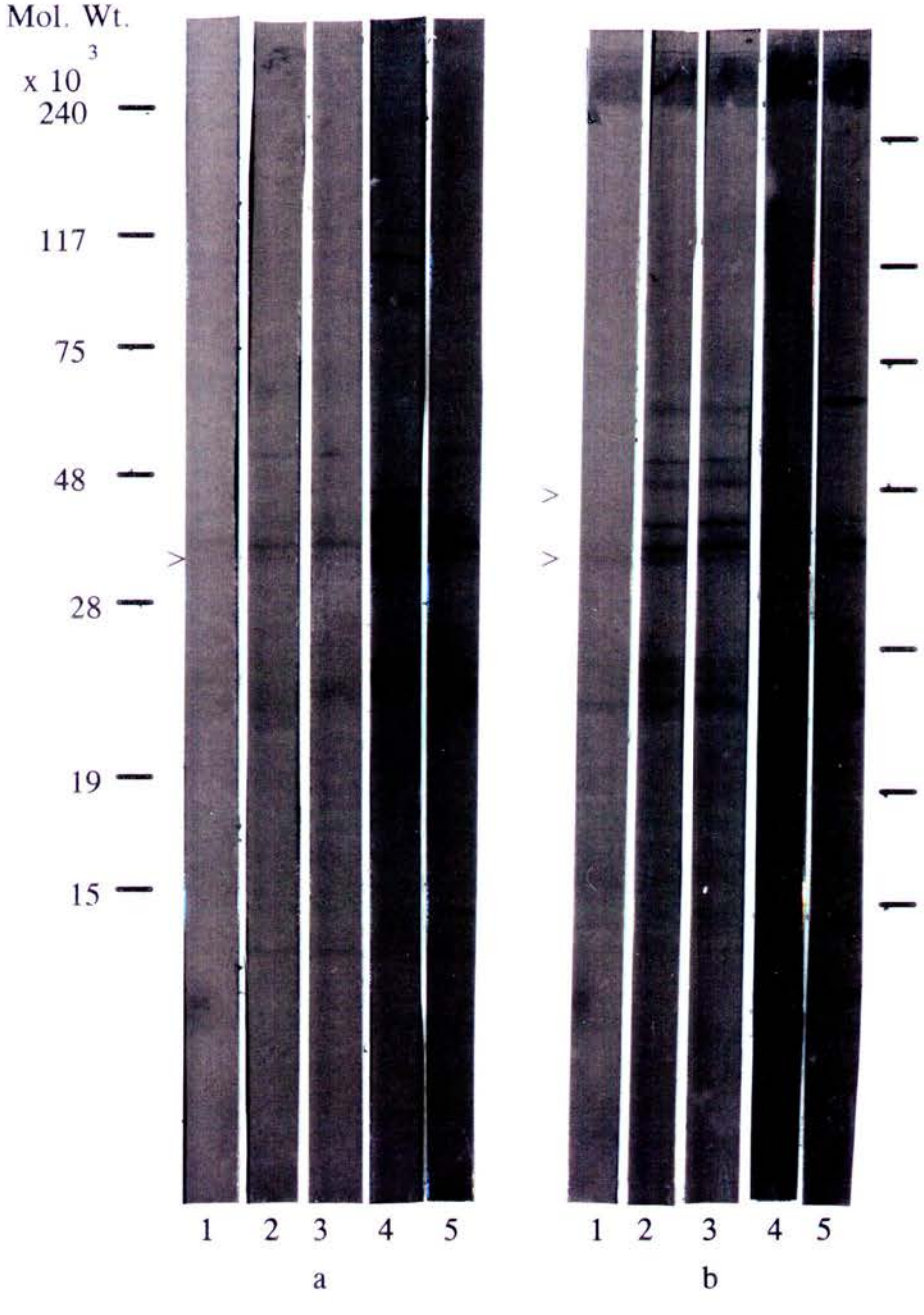


Figure 4.33.

Western blots of PBS-PI extracts of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

25 kDa was more strongly recognised and the 62 kDa was recognised as a doublet. Apart from all the antigens detected by sera collected earlier in the infection, D56 sera faintly detected a doublet of about 75 kDa (Figure 4.34).

Surface CTAB Extract of Adult Flukes

D0 and D14 sera did not react with any components in CTAB extract run under reducing conditions but D28, D42 and D56 sera recognised an antigen of about 25 kDa. When the extract was run under non-reducing conditions, D0 and D14 sera detected no antigens but D28 sera faintly recognised a doublet of about 48 kDa while D42 and D56 sera recognised an additional antigen of about 25 kDa. In addition, D56 sera also faintly recognised an antigen of about <15 kDa (Figure 4.35 and Table 4.6).

4.6.4.3 Probing parasite extracts with sera from rats that received antiserum in passive protection trial 2

Pooled sera obtained from sequential and final bleeds of rats that were injected with antiserum in passive protection trial 2 were used to probe ES_{14} and ES_A as well as NoG detergent extracts of adult flukes in order to check for transfer of potentially protective antibodies and to monitor the pattern of antibody development in these rats. Pooled sera from rats injected with antiserum but which had no flukes and no apparent liver lesions were also used to probe these ES preparations and extracts as well as ES_0. The ES products of D1 flukes were not used for probing because the amount available was too little.

ES Extracts of Flukes

Pooled sera collected from rats given immune serum I_2 1 and 3 days after infection recognised a 25 kDa doublet in ES_{14} run under reducing conditions. These antigens were also faintly recognised by D14 sera but not by D28 sera. However, the antigens were again detected by D42 and D56. A similar pattern was observed when the sera was used to probe the non-reduced form of this ES (Figure 4.36).

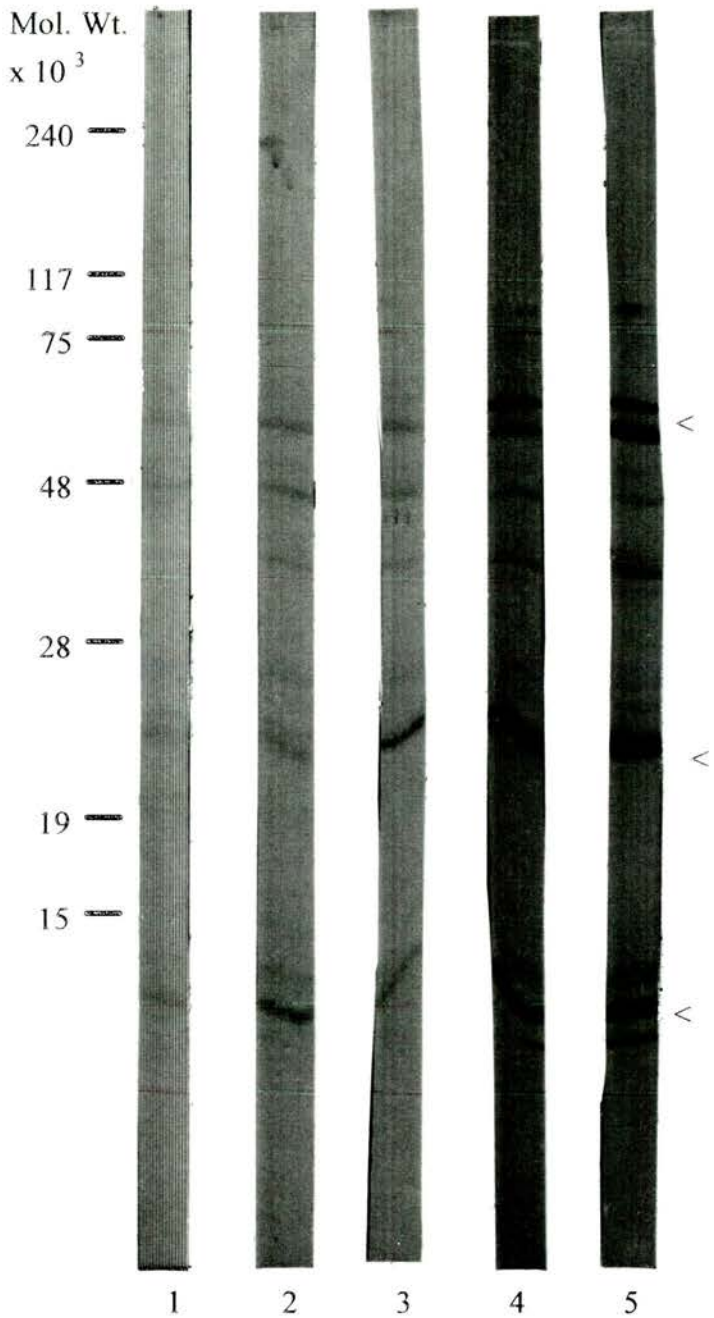


Figure 4.34.

Western blots of NoG surface detergent extracts of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gel was run under reducing conditions.

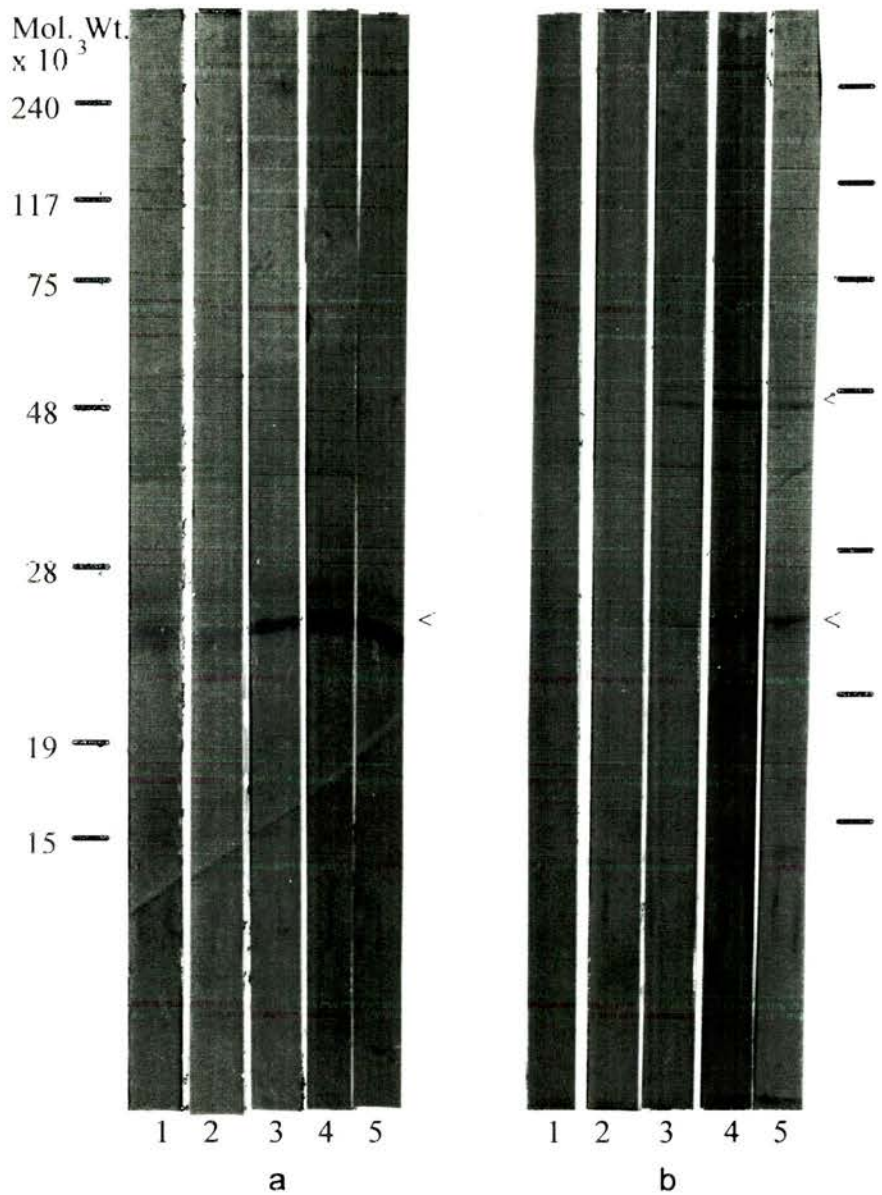


Figure 4.35

Western blots of CTAB detergent surface extracts of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3) D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gels were run under either reducing (a) or non-reducing conditions (b)

Table 4.6 Antigens recognised in ES and extracts of flukes by sera obtained from sequential bleeds of rats with flukes and pathology in trial 2

Extract	Day after infection at which sera was collected									
	0		14		28		42		56	
	R	NR	R	NR	R	NR	R	NR	R	NR
MED	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
ES_O	-ve	ND	-ve	ND	-ve	ND	191	ND	191	ND
ES_1	-ve	ND	-ve	ND	-ve	ND	191	ND	191	ND
ES_{14}	-ve	-ve	-ve	-ve	-ve	-ve	25	25	25	25
ES_A	-ve	-ve	-ve	-ve	-ve	-ve	25	27 26	25	27 26
PBS-PI	-ve	-ve	38	- 48 43 - 23	38	- 48 43 - 23	38 24 23 <15	- 43 24 23	38 24 23 <15	63 - 43 24 23 <15
CTAB	-ve	-ve	25	48	25	48	25	48 25	25	48 25 <15
NoG	-ve	-ve	- 62 48 38 25 <15	*	- 62 48 38 25 <15	*	- 62 48 38 25 <15	*	75 62 48 38 25 <15	* <15 <15

* Done twice but on both occassions only two antigens <15 kDa were detected
ND Not done

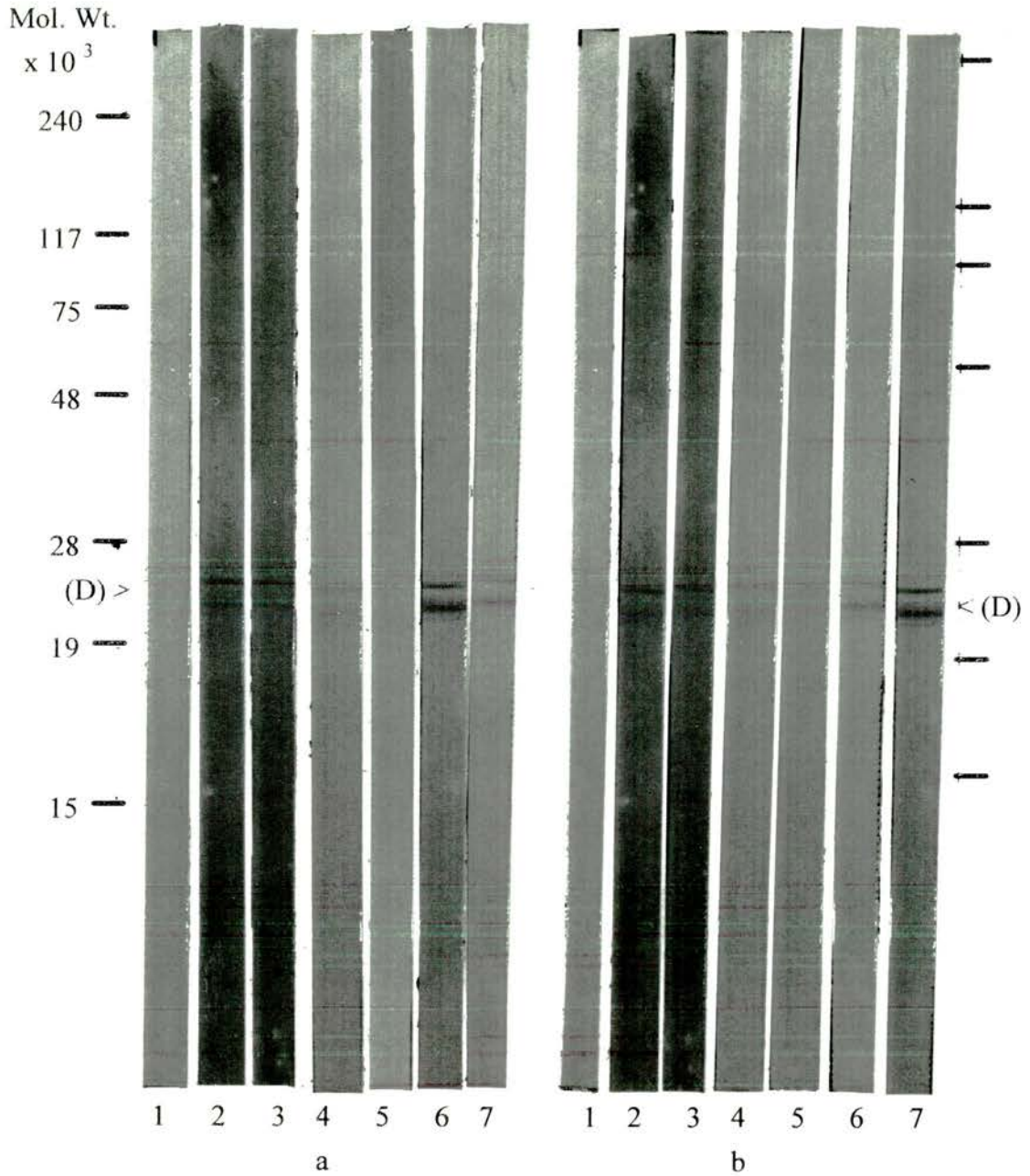


Figure 4.36.

Western blots of ES products of D14 *F. hepatica* probed with D0 (track 1), D1 (track 2), D3 (track 3), D14 (track 4), D28 (track 5), D42 (track 6) and D56 (track 7) sera from rats injected with antiserum in passive protection trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

Pooled sera collected from rats given immune serum I_2 1 and 3 days after infection recognised a 25 kDa doublet in ES_A run under reducing conditions. D14 and D28 sera still recognised these antigens but this recognition was very faint. These antigens were again strongly recognised by D42 and D56 sera. When the same serum was used to probe the non-reduced ES_A, one major antigen of about 26 kDa and a minor one with apparent weights of about 27 kDa were revealed. D14 and D28 sera recognised both antigens but this recogntion was very faint. The antigens were again strongly recognised by D42 and D56 sera (Figure 4.37).

NoG Extract of Adult Fluke

Five antigens with apparent weights of <15, 25, 38, 48 and 63 kDa were recognised in the reduced form of this extract by D1 and D3 sera. The 25 kDa was recognised as a major antigen. D14 sera did not recognise the 48 and 38 kDa antigens and only weakly recognised the other three antigens. D28 sera recognised only the <15 kDa antigen while D42 and D56 sera both strongly recognised the 25 and <15 kDa antigens (Figure 4.38 and Table 4.7).

Probing Parasite Extracts With Sera From Rats Injected With Antiserum But Which Had No Flukes Or Pathology At Necropsy In Passive Protection Trial 2

Pooled D1 and D3 sera from these rats strongly recognised the 191 kDa antigen in ES_0 run under reducing conditions. Although D14 and D28 sera still recognised this antigen, the recognition was progressively weaker. D42 and D56 sera did not recognise this antigen (Figure 4.39).

The doublet with an apparent weight of about 25 kDa was detected by pooled D1 and D3 sera in ES_{14} run under reducing conditions but notably, sera collected subsequently did not recognise these antigens at all. A similar pattern was observed following probing of the non-reduced form of this ES (Figure 4.40).

D1 and D3 sera recognised the 25 kDa doublet and a minor antigen with molecular weight of <15 kDa in ES_A run under reducing conditions. Notably, the recognition of these antigens became weaker as the infection progressed and sera

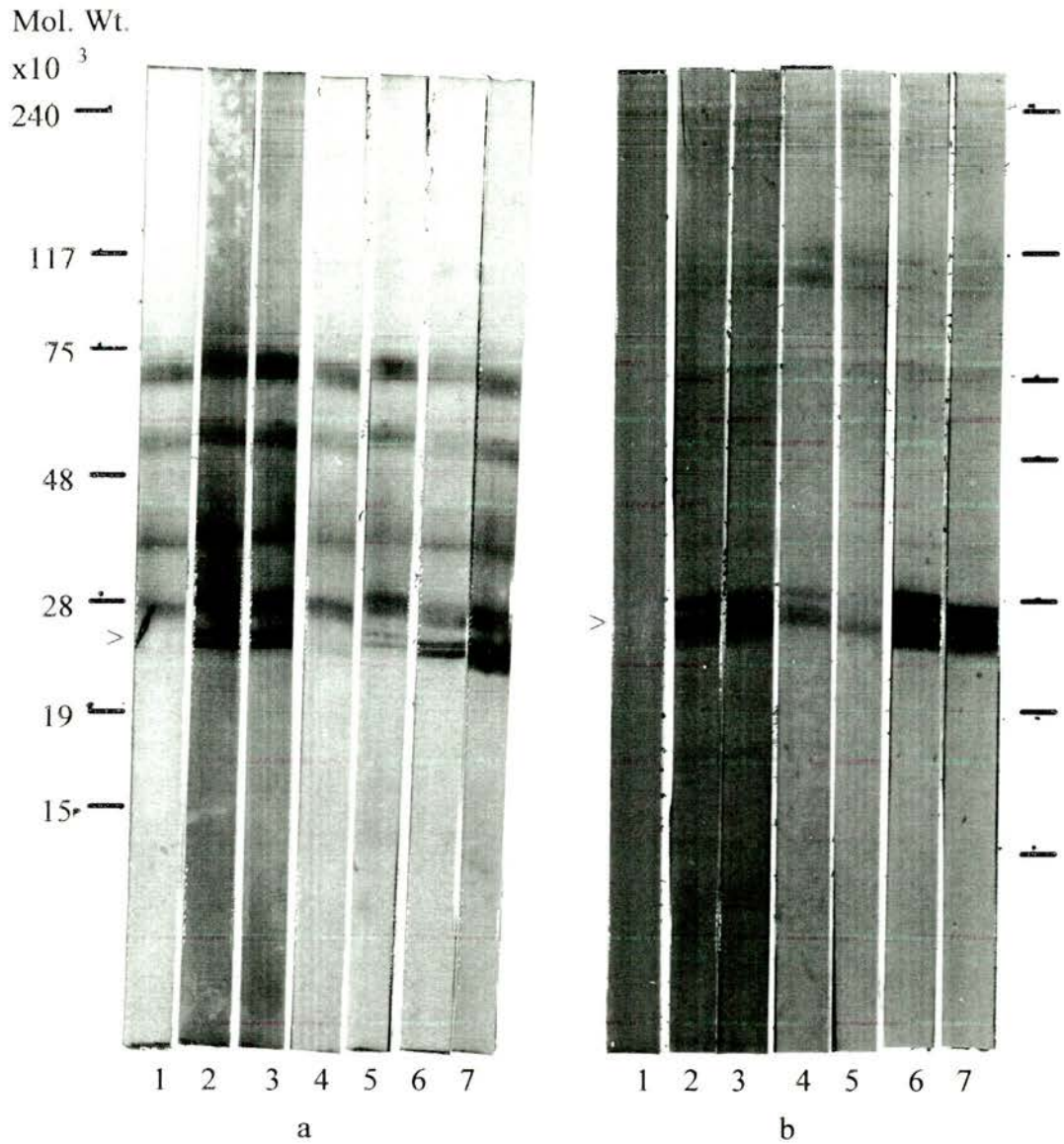


Figure 4.37.

Western blots of ES products of adult *F. hepatica* probed with D0(track 1), D1 (track 2), D3 (track 3), D14 (track 4), D28 (track 5), D42 (track 6) and D56 (track 7) sera from rats injected with antiserum in passive protection trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

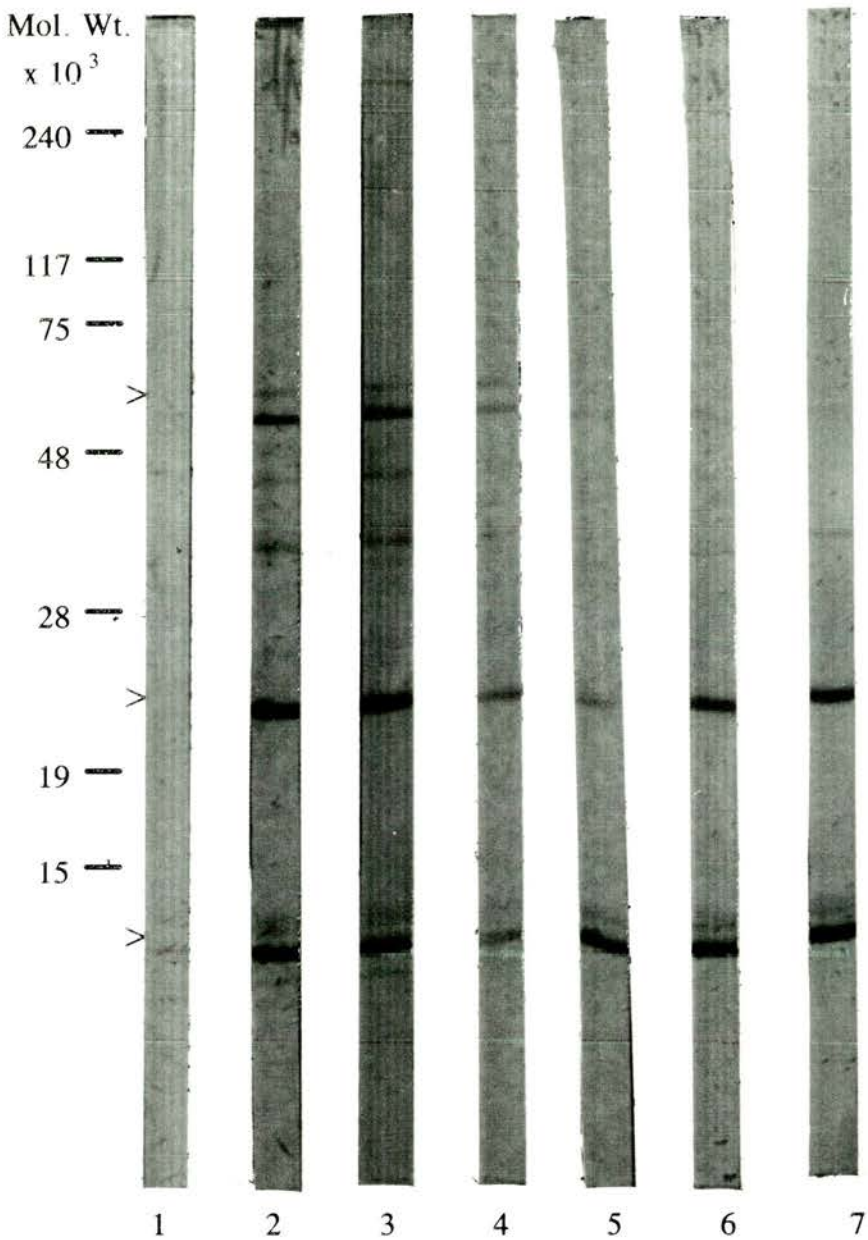


Figure 4.38.

Western blots of NoG extracts of adult *F. hepatica* probed with D0 (track 1), D1 (track 2), D3 (track 3), D14 (track 4), D28 (track 5), D42 (track 6) and D56 (track 7) sera from rats injected with antiserum in passive protection trial 2. Gel was run under reducing conditions.

Table 4.7 **Antigens recognised in ES_{14}, ES_A and NoG extract of adult fluke by sera obtained from rats injected with antiserum in passive protection trial 2**

Extract	Day after infection at which sera was collected											
	1		3		14		28		42		56	
	R	NR	R	NR	R	NR	R	NR	R	NR	R	NR
ES_{14}	25	25	25	25	25	25	-ve	-ve	25	25	25	25
ES_A	25	27 26	25	27 26	25	27 26	-ve	-ve	25	27 26	25	27 26
NoG	63 48 38 25 <15	ND	63 48 38 25 <15	ND	63 - - 25 <15	ND	- - - 25 <15	ND	- - - 25 <15	ND	- - - 25 <15	ND

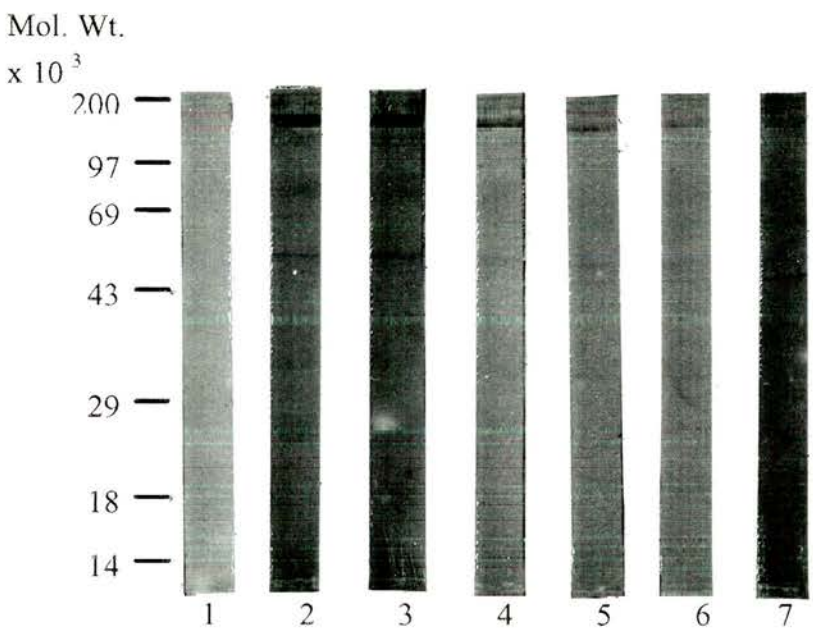


Figure 4.39.

Western blots of ES products of D0 *F.hepatica* probed with D0 (track 1), D1 (track 2), D3 (track 3), D14 (track 4), D28 (track 5), D42 (track 6) and D56 (track 7) sera from rats injected with antiserum but which had neither flukes nor pathology in passive protection trial 2. Gel was run under reducing conditions.

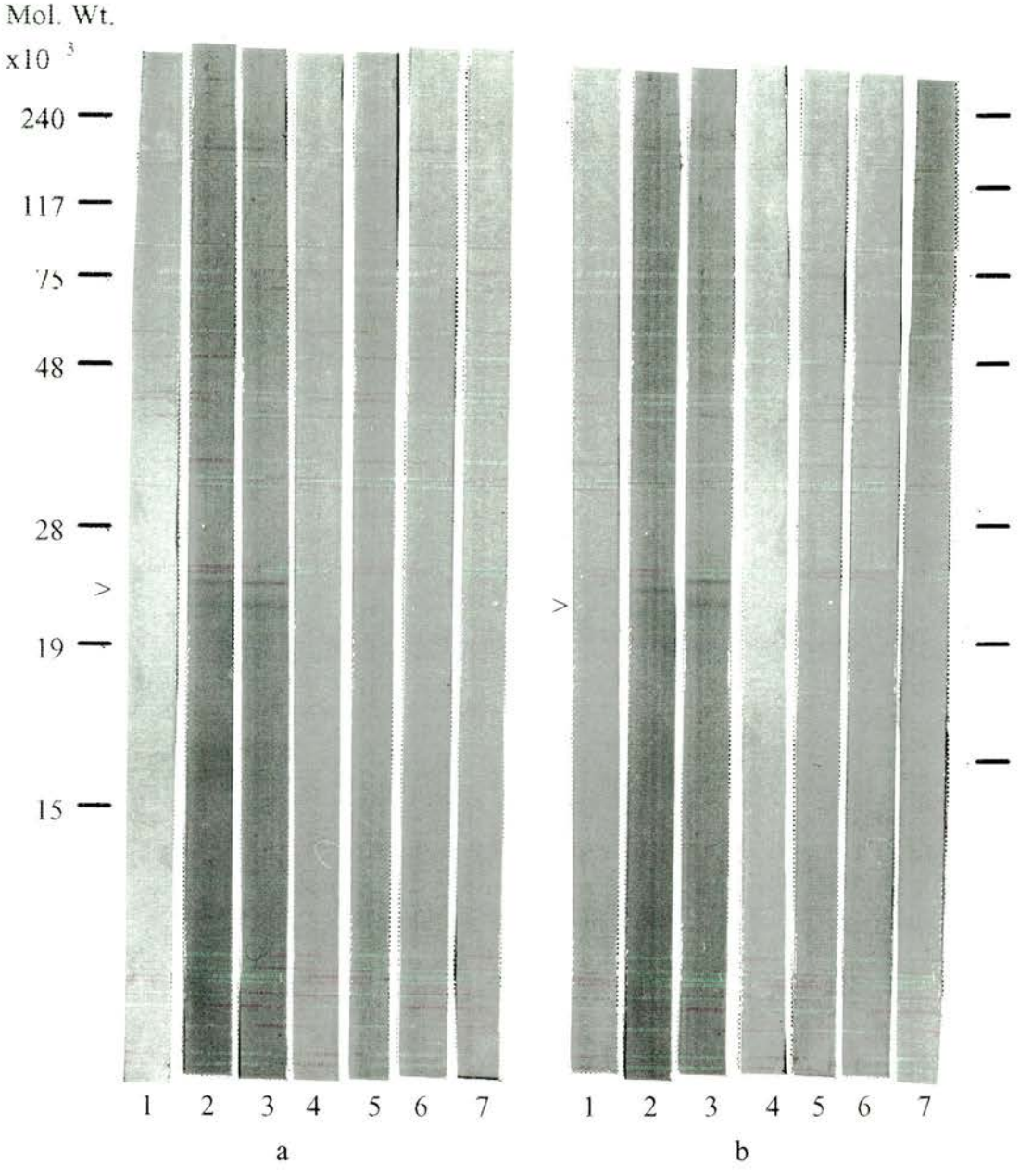


Figure 4.40.

Western blots of ES products of D14 *F. hepatica* probed with D0 (track 1), D1 (track 2), D3 (track 3), D14 (track 4), D28 (track 5) D42 (track 6) and D56 (track 7) sera from rats injected with antiserum but which had neither fluke nor pathology in passive protection trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

obtained by D56 of infection did not recognise these antigens. A similar pattern was observed when the sera were used to probe the non-reduced form of ES_A. Pooled D1 and D3 sera also recognised antigens with apparent weights of 26 and 27 kDa in this extract. The recognition of these antigens again notably decreased as the infection progressed, such that by D56 the 27 kDa antigen was only very weakly recognised (Figure 4.41).

D1 and D3 sera recognised antigens with apparent weights of 63, 48, 38, 25 and <15 kDa in the reduced form of NoG extract. The 25 kDa was a major antigen. D14 and D28 sera did not recognise the 63, 48 and 38 kDa antigens. However, D28 sera faintly recognised the 25 and <15 kDa antigens. Neither D42 nor D56 sera recognised the 25 kDa antigen (Figure 4.42).

4.6.4.4 Probing ES_A with sera collected from rats used in passive protection trial 1

ES_A was probed with sera obtained from rats given antiserum in the first passive protection trial in order to monitor the pattern of antibody development in these rats.

The 25 kDa doublet in ES_A (reducing conditions) was recognised by D14 sera. The recognition of these antigens was weaker when the ES was probed with D28 sera. D42 and D56 sera both strongly recognised these antigens again. A similar pattern was observed with ES that was run under non-reducing conditions (Figure 4.43).

4.6.4.5 Probing adult fluke extract with sera from rats used in trial 3 to produce antiserum

Sera (I_3) collected following exsanguination of the rats used in this trial significantly protected recipient rats against oral challenge. This serum, as well as sera collected earlier in the infection, were used to probe ES and the somatic extract of adult flukes in order to monitor the pattern of antibody development.

D14 and D28 sera recognised two antigens (25 kDa doublet and <15 kDa) in ES_A (reduced) as well as two antigens of about 25 and 26 kDa (non-reduced). D42

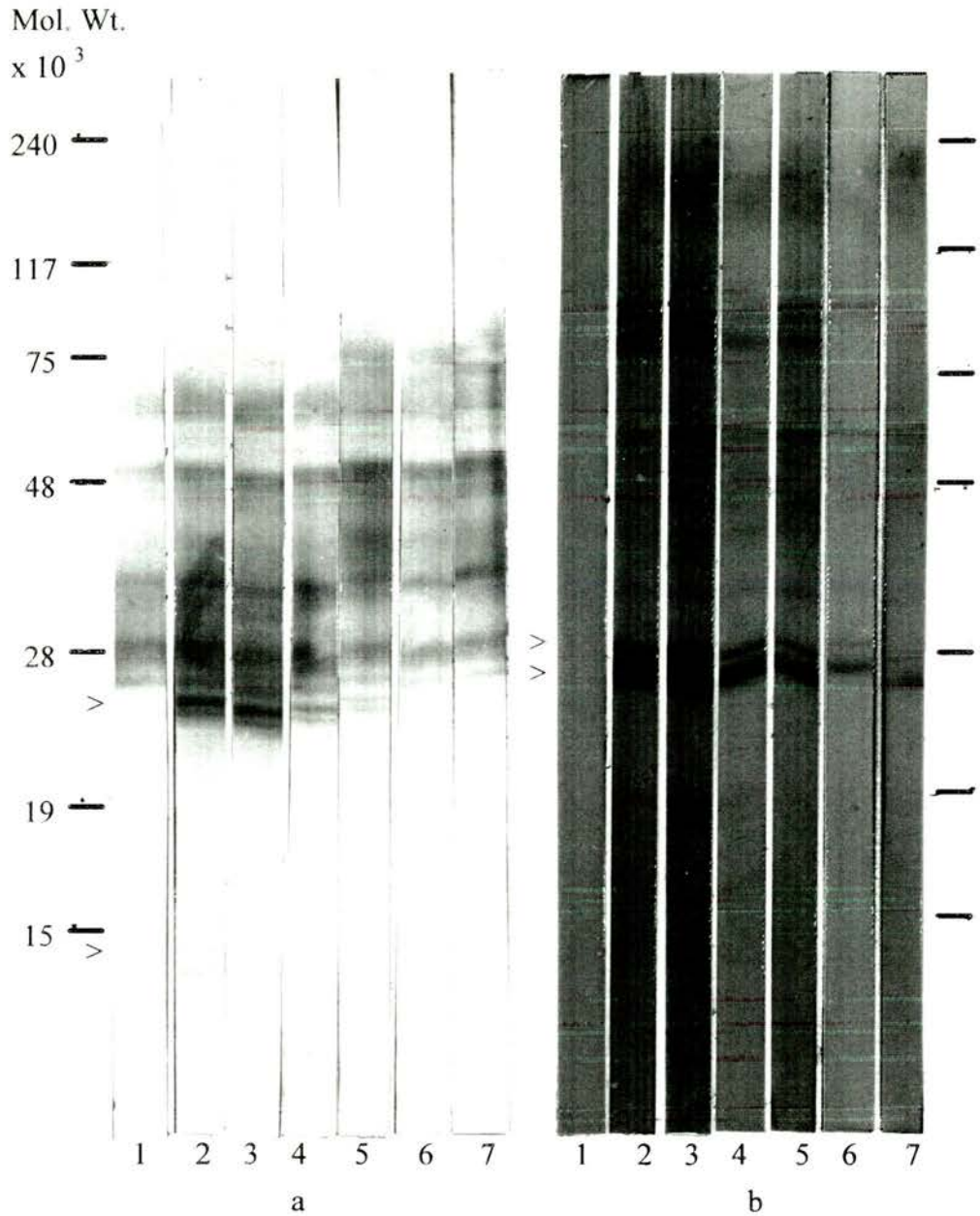


Figure 4.41.

Western blots of ES products of adult *F. hepatica* probed with D0 (track 1), D1 (track 2), D3 (track 3), D14 (track 4), D28 (track 5), D42 (track 6) and D56 (track 7) sera from rats injected with antiserum but which had neither flukes nor pathology in passive protection trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

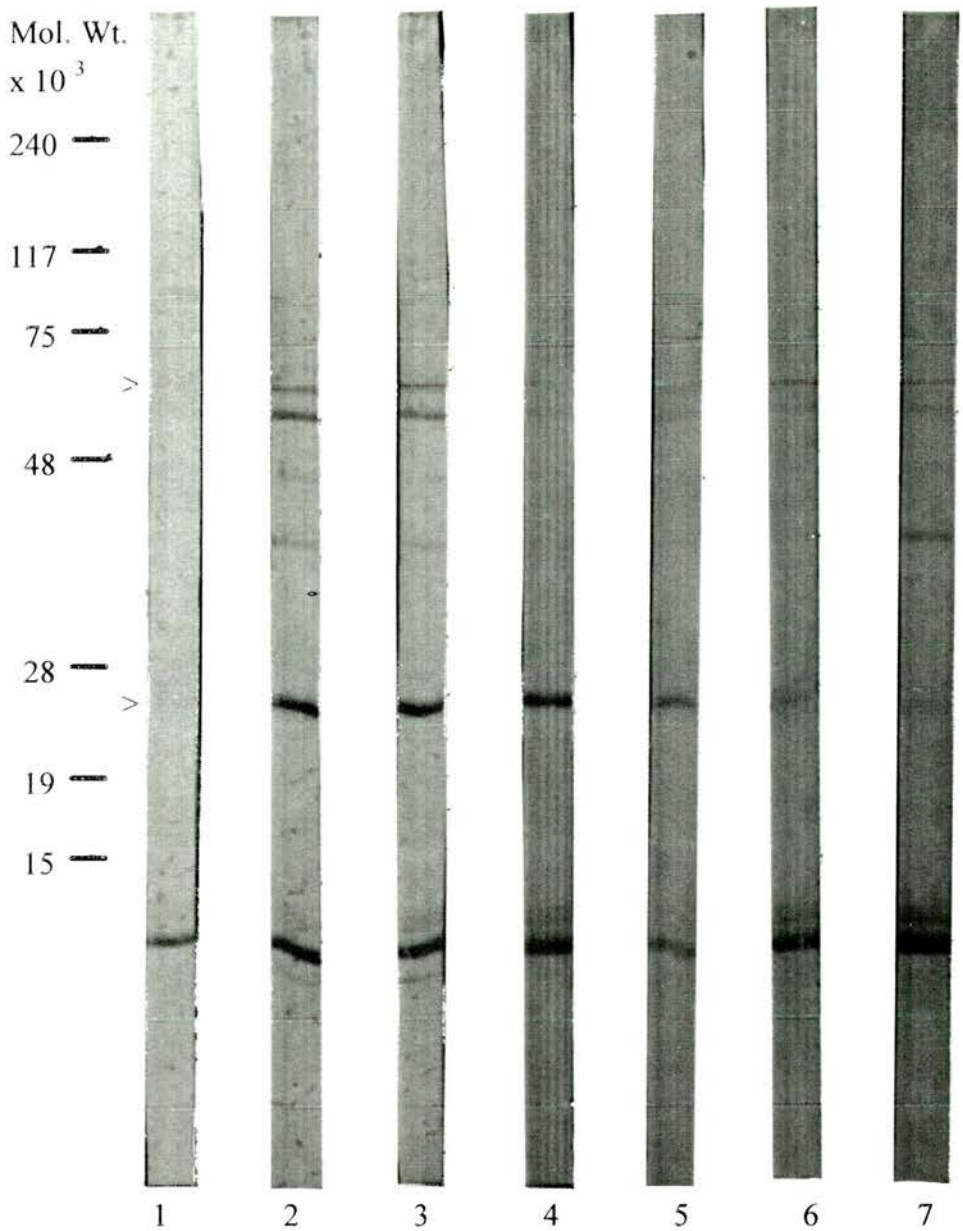


Figure 4.42.

Western blots of surface NoG extracts of adult *F. hepatica* probed with D0 (track 1), D1 (track 2), D3 (track 3), D14 (track 4), D28 (track 5), D42 (track 6) and D56 (track 7) sera from rats injected with antiserum but which had neither flukes nor pathology in passive protection trial 2. Gel was run under reducing conditions.

Table 4.8 **Antigens recognised in ES_0, ES_{14}, ES_A and NoG extract of adult fluke by sera obtained from rats injected with antiserum but which had neither flukes nor pathology in passive protection trial 2**

Extract	Day after infection at which sera was collected											
	1		3		14		28		42		56	
	R	NR	R	NR	R	NR	R	NR	R	NR	R	NR
ES_0	191	25	191	ND	191	ND	191	ND	-ve	ND	-ve	ND
ES_{14}	25	25	25	25	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
ES_A	25	27	25	27	25	27	25		25	-	-ve	-
	<15	26	<15	26	-	26	-		-	26	-	-
NoG	63	ND	63	ND	-	ND	-	ND	-	ND	-	ND
	48		48		-		-		-		-	
	38		38		-		-		-		-	
	25		25		25		25		-		-	
	<15		<15		<15		<15		<15		<15	

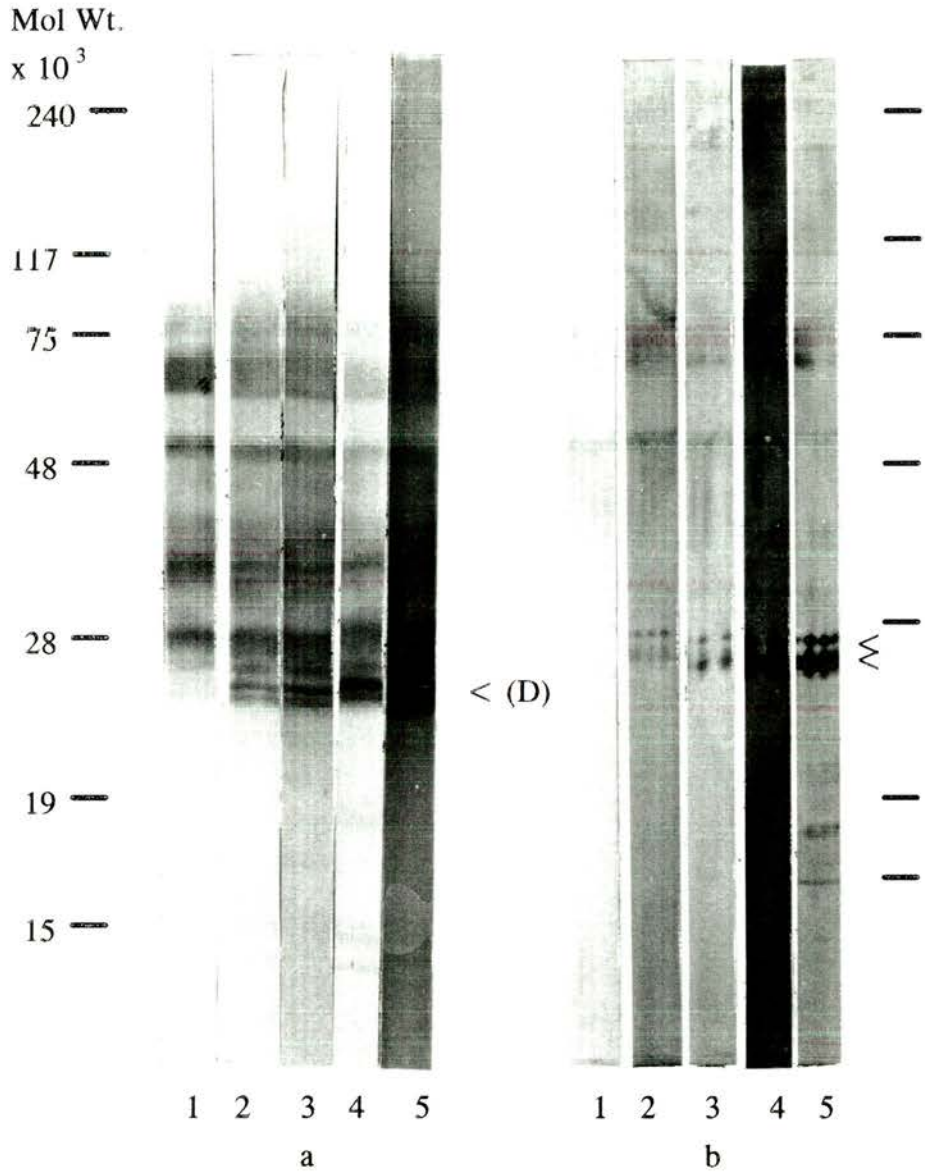


Figure 4.43.

Western blots of ES products of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats injected with antiserum in passive protection trial 1. Gels were run under either reducing (a) or non-reducing (b) conditions.

Sera also recognised these antigens. An additional antigen (68 kDa) was recognised by this serum in ES_A (non-reduced). D56 serum detected three additional antigens of 20, 17 and <15 kDa in the non-reduced ES_A (Figure 4.44 and Table 4.9).

D14 sera recognised a 53 kDa doublet in reduced adult PBS-PI somatic extract. An additional 25 kDa doublet antigen was faintly recognised by D28 serum. D42 and D56 sera both recognised two additional antigens, a doublet of <15 kDa and an antigen of about 62 kDa. When this extract was run under non-reducing conditions, a 53 kDa antigen was recognised by D14 and D28 sera. Two additional antigens of about 43 and 24 kDa were recognised by D42 sera, while D56 sera detected yet an additional <15 kDa doublet antigen (Figure 4.45 and Table 4.9).

4.6.4.6 Probing western blots of ES_A with pooled total sera obtained from sequential bleeds of rats injected with antiserum in passive protection trial 3

Pooled D1 and D3 sera specifically recognised a doublet of about 25 kDa in ES_A run under reducing conditions. These antigens were still detected, though weakly, by D14 and D28 sera but very strongly by D42 and D56 sera. An additional antigen with an apparent weight of <15 Da was also detected by D56 serum.

D1 and D3 sera detected antigens of about 75, 27, 26, 20 and 17 kDa in the non-reduced ES_A. These antigens were also detected by D14 and D28 sera but D42 and D56 sera only faintly detected the 17 and 20 kDa antigens. (Figure 4.46).

Probing Western Blots of ES_A With Sera From Rats With Pathology But No Flukes

Gel profiles using reduced extracts were very similar to that described when total pooled sera was used. Some differences were noticed when this sera was used to probe ES_A run under non-reducing conditions. D42 serum only recognised the 27 and 26 kDa antigens. While D56 serum also recognised these two antigens and faintly recognised the 20 kDa antigen (Figure 4.47).

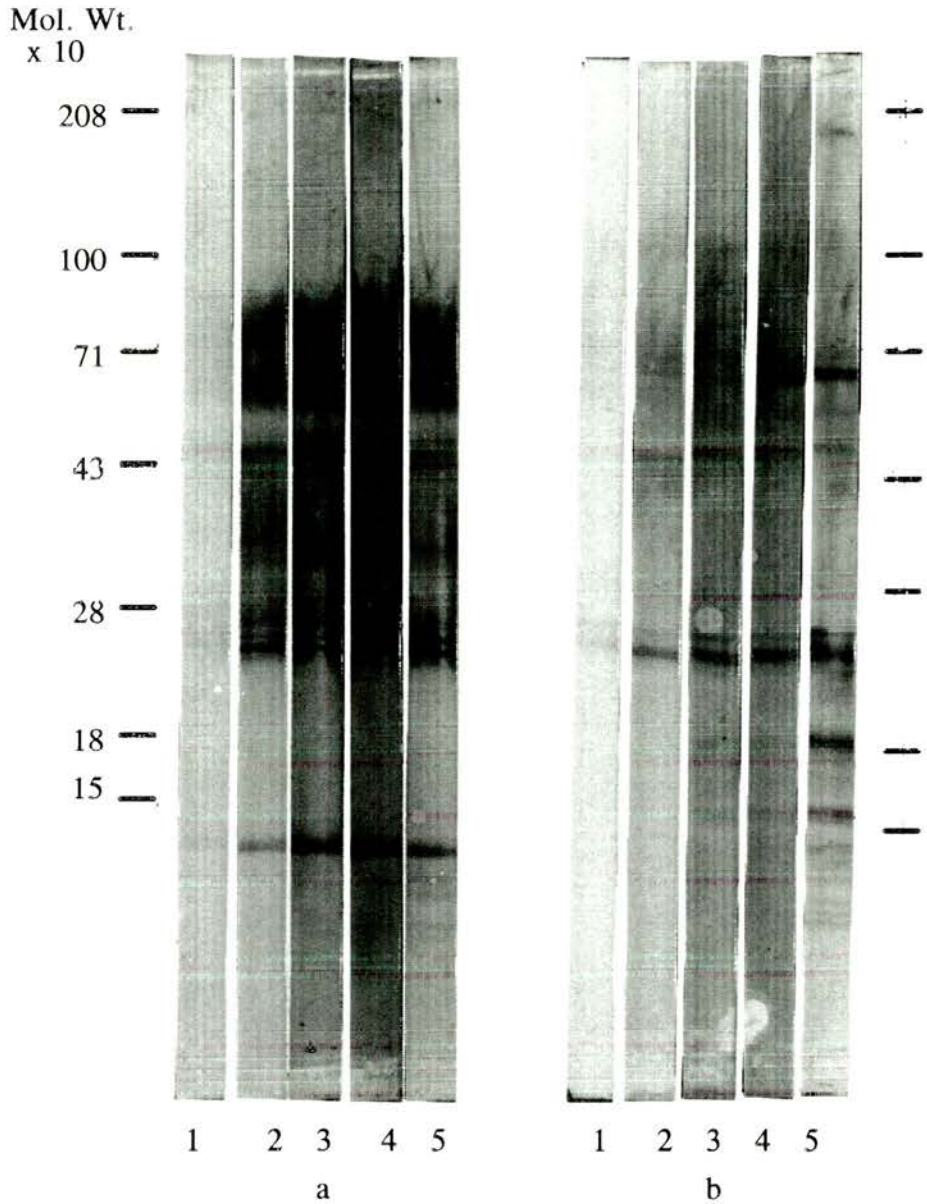


Figure 4.44.

Western blots of ES products of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 3. Gels were run under either reducing (a) or non-reducing (b) conditions.

Table 4.9 **Antigens specifically recognised in ES and somatic (PBS-PI) extract of adult fluke by sera obtained from rats with flukes and pathology in trial 3**

Extract	Day after infection at which sera was collected									
	0		14		28		42		56	
	R	NR	R	NR	R	NR	R	NR	R	NR
ES_A	-ve	-ve	25	-	25	-	25	68	25	68
			<15	26	<15	26	<15	26	<15	26
				25		25		25		25
				-		-		-		20
				-		-		-		17
				-		-		-		<15
PBS-PI			-		-		62		-	
	-ve	-ve	53	53	53	53	53	53	53	53
			-	-	25	-	25	43	25	43
				-		-		-		<15

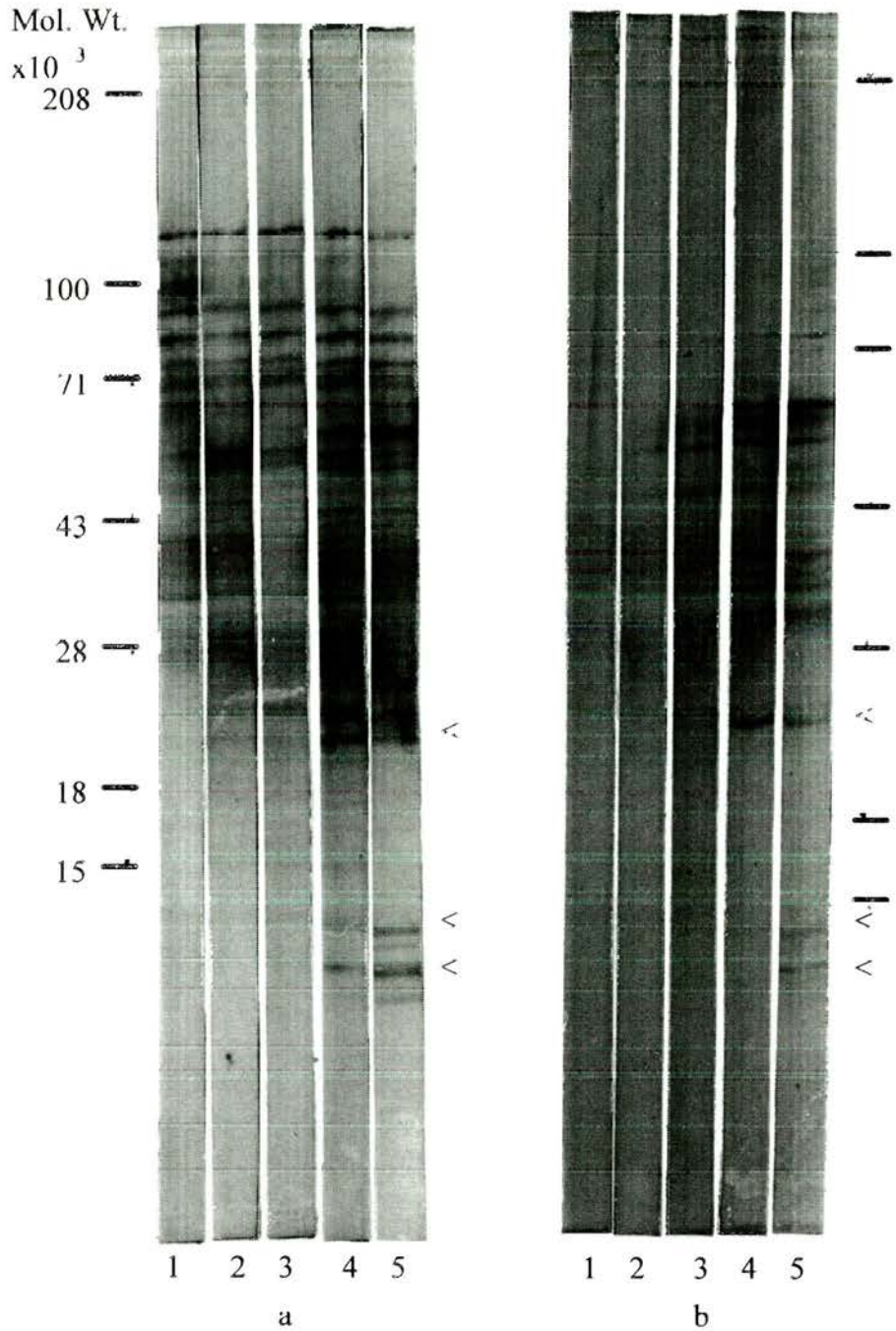


Figure 4.45.

Western blots of PBS-PI extracts of *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 3. Gels were run under either reducing (a) or non-reducing (b) conditions.

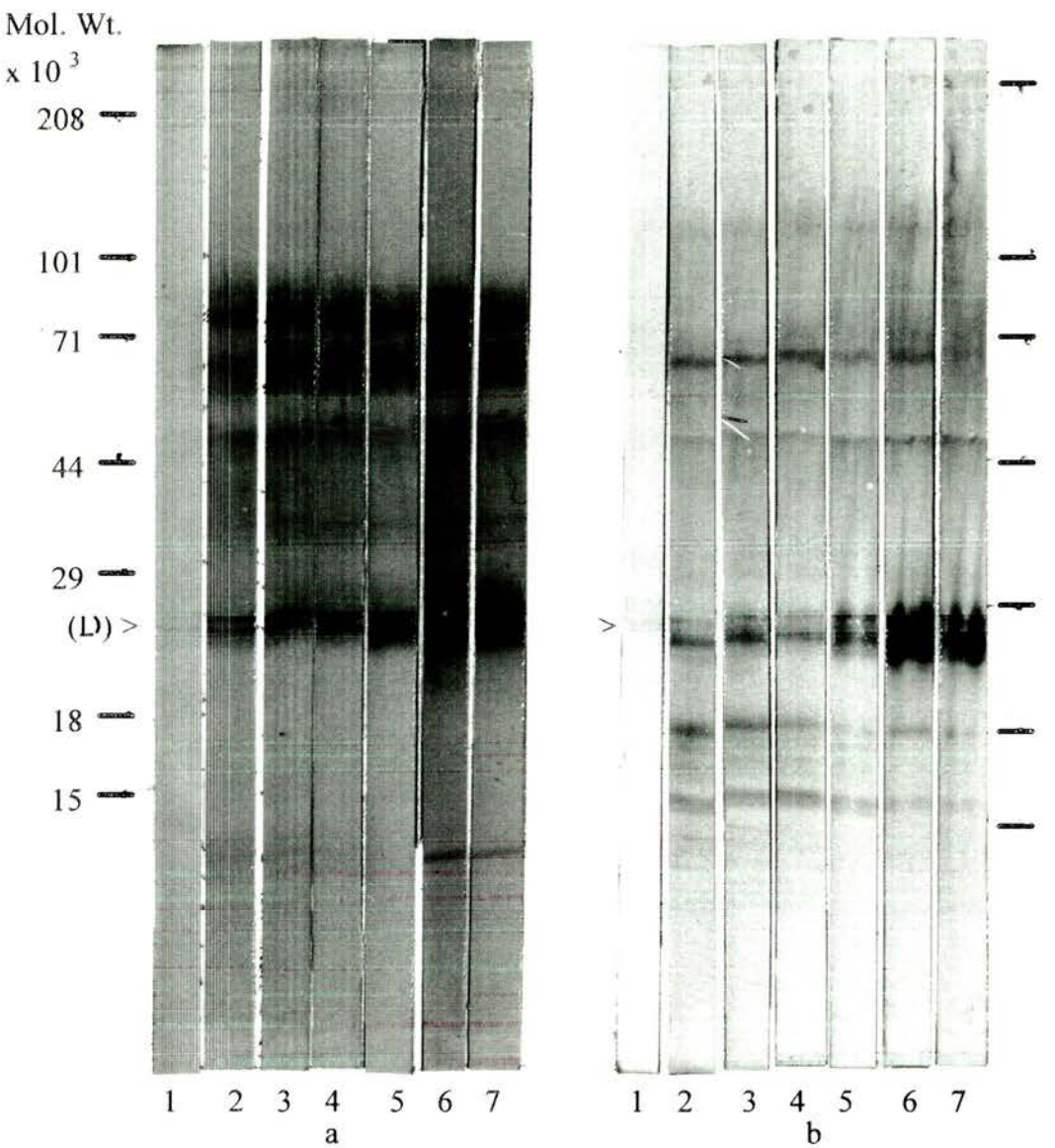


Figure 4.46

Western blots of ES products of adult *F. hepatica* probed with D0 (track 1), D1 (track 2), D3 (track 3), D14 (track 4), D28 (track 5), D42 (track 6) and D56 (track 7) sera from rats injected with antiserum in passive protection trial 3. Gels were run under either reducing (a) or non-reducing (b) conditions.

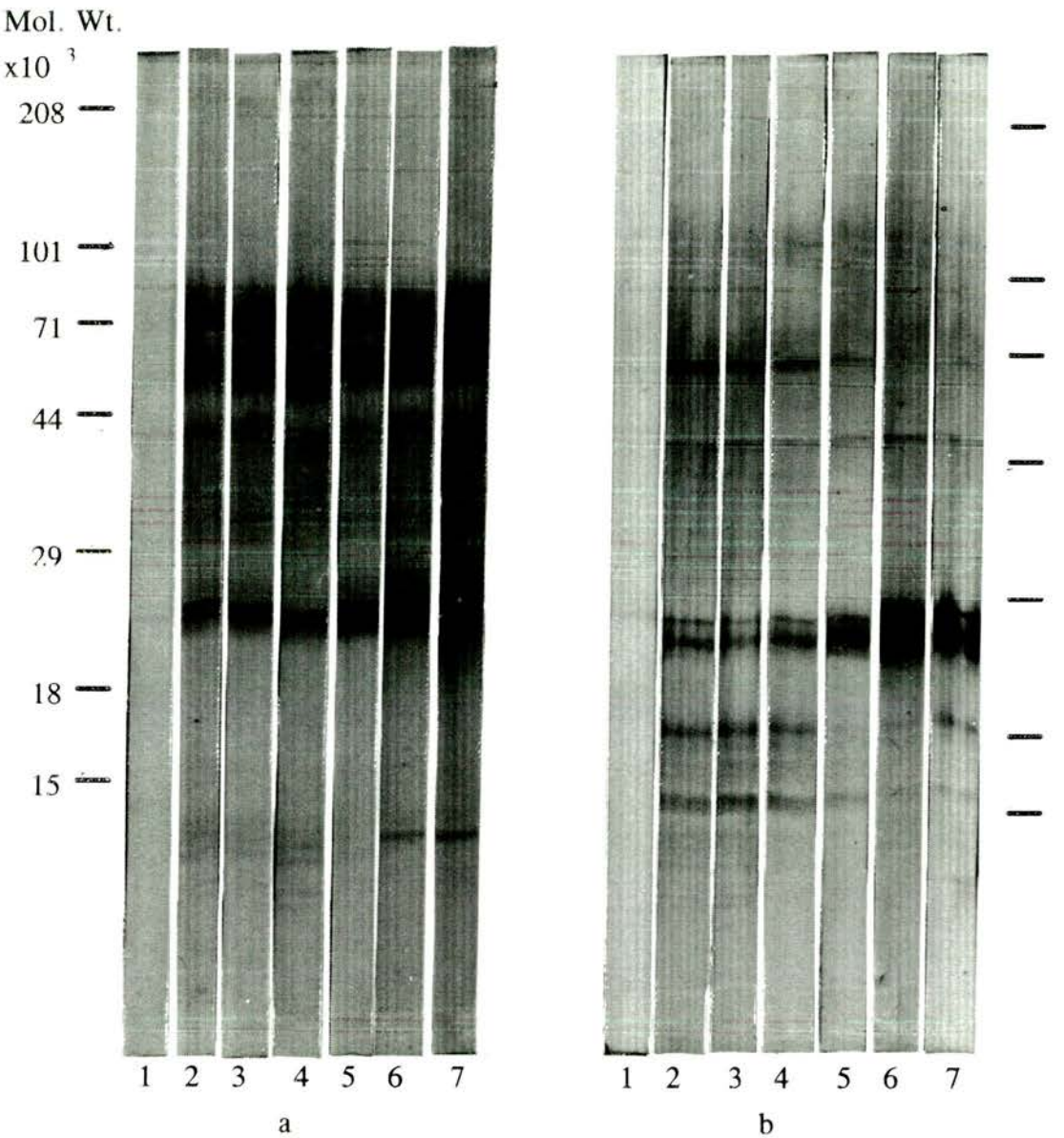


Figure 4.47.

Wetern blots of ES products of adult *F. hepatica* probed with D0 (track 1), D1 (track 2), D3 (track 3), D14 (track 4) D28 (track 5), D42 (track 6) and D56 (track 7) sera from rats injected with antiserum but which had neither flukes nor pathology in passive protection trial 3. Gels were run under either reducing (a) or non-reducing (b) conditions.

4.7 STUDIES ON ES AND SOMATIC PROTEINS OF FLUKES BIOSYNTHETICALLY LABELLED WITH ^{35}S-METHIONINE

Excretory/secretory proteins of various developmental and adult stages of *F. hepatica* were biosynthetically radio-labelled, the labelling being repeated twice for all expect D28 and D42 flukes. Somatic extracts of biosynthetically radio-labelled D28, D42 and adult flukes were prepared in PBS-PI-NP40.

4.7.1 Total Biosynthetically Radio-labelled Proteins in ES Products of Flukes

The total radio-labelled ES proteins of the various stages were compared, and their profiles are presented in Figure 4.48 (a and b) and Table 4.10 (a and b) summarises the sizes of the main components.

The biosynthetically radiolabelled ES of various ages of flukes showed noticeable similarities and differences. In the reduced state, D0 flukes had the simplest profile, with only three minor components. In contrast, the ES of D1 flukes (ES_1) contained an array of components. All the components of D0 ES (ES_0) were present in ES_1 but there were nine new components. A component of about 39 kDa in ES_{14} was possibly an analogue of the 36 kDa component in ES_1. The component of about 30 kDa in ES_1 was expressed as a major component with an apparent molecular weight of about 26-30 kDa in ES_{14}. All the remaining new components that were detected in ES_1 as well as another component of about 27 kDa were not detectable in ES_{14}. ES_{14} was quite similar to the ES products of D28 flukes (ES_{28}) but few differences were apparent. One new, moderately strong, component of about 16 kDa was expressed in ES_{28}. ES_{42} was again similar to ES_{28}. However, a new component of 119 kDa was expressed by D42 flukes. Another new component of about 14 kDa may be the same as the 16 kDa component in ES_{28}. Slight differences could be noticed in the ES of D42 and adult flukes (ES_A). A moderately expressed component of about 40 kDa as well as a minor component of about 119 kDa in the former were not present in the latter. Conversely, minor components of about 87, 93 and 107 kDa detected in the latter were absent in the former.

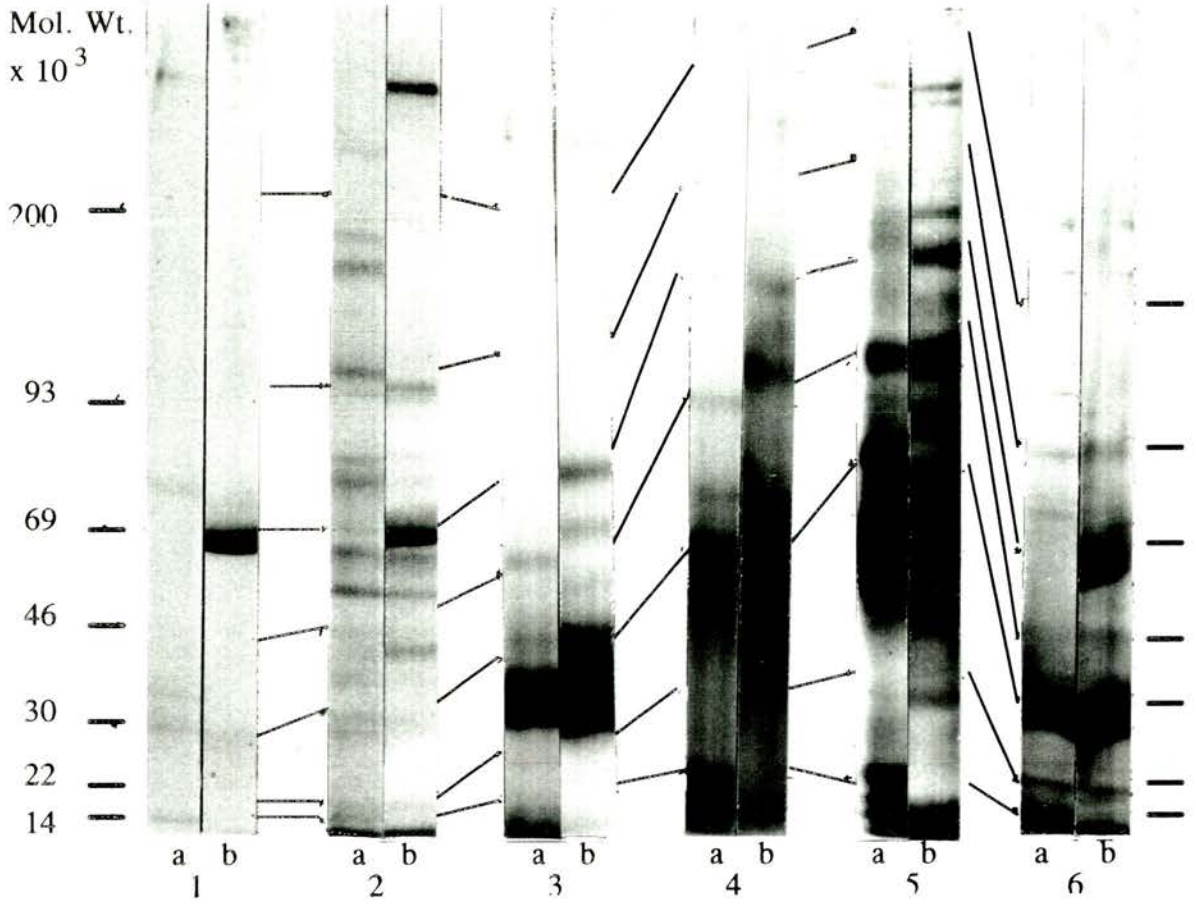


Figure 4.48a.

Total biosynthetically radio-labelled ES proteins of D0 (track 1), D1 (track 2), D14 (track 3), D28 (track 4), D42 (track 5) and D56 (track 6) *F. hepatica*. Gels were run under either reducing (a) or non-reducing (b) conditions.

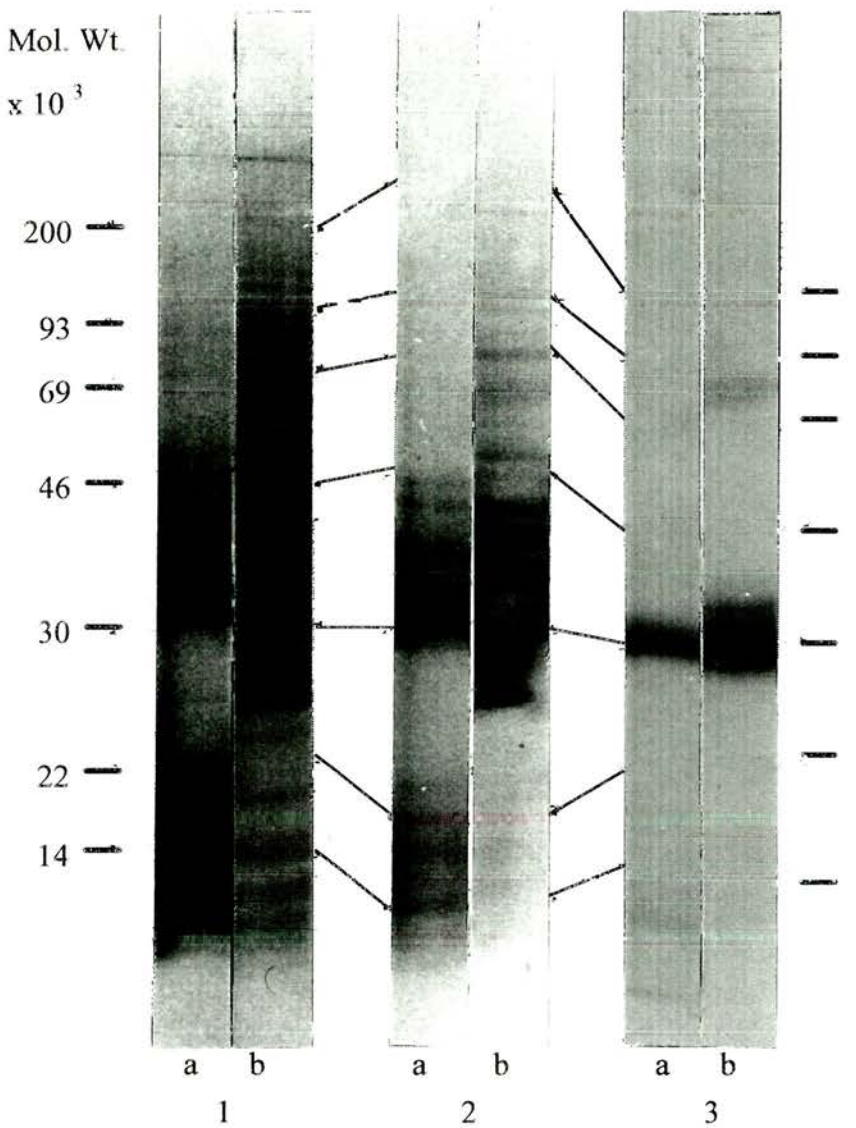


Figure 4. 48b.

Total biosynthetically radiolabelled proteins in PBS-PI-NP40 extracts of D28 (track 1), D42 (track 2) and D56 (track 3) *F. hepatica*. Gels were run under either reducing (a) or non-reducing (b) conditions

Table 4.10a Apparent molecular weights of proteins detected in biosynthetically (^{35}S-methionine) radio-labelled ES products of flukes run under reducing conditions

Age of fluke in days					
0	1	14	28	42	56
-	168	-	-	-	-
-	155	-	-	-	-
-	-	-	-	119	-
-	-	-	-	-	107
-	103	-	-	-	-
-	-	-	-	-	93
-	-	-	-	-	87
-	82	-	-	-	-
-	78	-	-	-	-
76	-	-	-	-	-
-	54	-	-	-	-
-	46	46	46	46	46
-	-	-	-	40	-
-	-	39	39	-	-
-	36	-	-	-	-
-	30	-	-	-	-
-	-	-	-	28-30	-
27	27	-	27-30	-	27-30
-	-	26-30	-	-	-
22	-	-	-	-	-
-	21	-	-	-	21
-	-	-	16	-	-
-	-	-	-	14	14
-	-	-	<14	<14	-

Table 4.10b Apparent molecular weights of proteins detected in biosynthetically (^{35}S-methionine) radio-labelled ES products of flukes run under non-reducing conditions

Age of fluke in days					
0	1	14	28	42	56
-	>200	-	-	-	-
-	-	-	-	119	-
-	92	-	-	-	92
-	82	-	-	-	-
-	-	-	-	79	-
-	77	-	-	-	-
69	69	69	69	69	-
-	-	-	-	-	62
-	55	-	-	-	-
-	46	46	46	46	46
-	40	-	-	-	-
-	-	36	36	36	-
30	-	-	-	-	-
-	28	-	-	-	-
-	-	-	26-30	26-30	26-30
-	-	23-30	-	-	-
21	21	-	-	21	21
-	-	-	-	<14	-

When the ES were run under non-reducing conditions, variations and similarities were still apparent. Again, ES_0 had the simplest profile, consisting of one major (69 kDa) and two minor (30 and 21 kDa) components. The minor components were both detected in ES_1 and the 69 kDa component may be an analogue of a major component of about 69 kDa in ES_1. Apart from these similarities, five new components were seen in ES_1. That of >200 kDa was a major component. The ES_{14} did not contain the 21, 55, 75 and >200 kDa components but it contained all other components detected in ES_1, although the molecular weights were not always exactly the same. The component with an apparent weight of 23-30 in ES_{14} was expressed as a major component while the 46 and 69 kDa components were moderately well represented. There were only slight differences between ES_{14} and ES_{28}. The 69 kDa component was more strongly expressed in ES_{14}, while the 46 kDa component was more strongly expressed in ES_{28}. ES_{28} was again quite similar to ES_{42} although there were minor differences. A new component of about 119 kDa was detected in ES_{42}. This component (119 kDa) and another of about 36 kDa were not detected in ES_A.

4.7.2 Total Biosynthetically Radio-labelled Proteins in Somatic (PBS-PI-NP40) Extracts of Flukes

The profiles of PBS-PI-NP40 extracts of biosynthetically radio-labelled D28 and D42 flukes were quite similar. When run in the reduced state, the only apparent differences were two components of about 46 kDa in the extract of D42 flukes, which were absent in the D28 extract. Fewer components were detected in the extract of adult fluke. Similar sized components were detected in the extracts of the other two ages of fluke.

In the non-reduced state, there were still similarities in the profiles of D28 and D42 fluke extracts. However, two moderately expressed components (146 and 134 kDa) as well as four minor components within the <14 to 21 kDa range in the extract of D28 fluke were not present D42 fluke extract. A major component of 28-

30 kDa detected in the extract of adult fluke was close in size to the 31-35 kDa component in the extracts of D28 and D42 fluke. A second component of about 72 kDa in the non-reduced adult fluke extract was not present in the extract of D42 fluke but could be similar to a faintly expressed minor component of about 70 kDa in the extract of D28 fluke.

The apparent weight of all components detected in these extracts are presented in Table 4.11.

4.7.3 Immuno-coprecipitation Studies Using Sera Obtained From Trial 1

Sera obtained from sequential and final bleeds of rats that had flukes and liver pathology or liver pathology without flukes in trial 1 (rats used to produce transferred serum in trial 1) was was used to probe the biosynthetically labelled ES proteins of adult flukes.

D14 serum from rats with flukes and pathology in trial 1 faintly detected an antigen of about 39 kDa as did sera obtained subsequently. The antigen was not detected in ES that was run under non-reducing conditions but an antigen of about 68 kDa was detected by D56 serum (Figure 4.49).

Sera obtained at D28 and subsequently from rats with liver pathology but no flukes faintly recognised an antigen of about 37 kDa in ES that was run under reducing but not under non-reducing conditions. This recognition was again very faint (Figure 4.50).

Sera obtained from rats that had neither flukes nor pathology did not specifically recognise any antigen in ES_A run under either reducing or non-reducing conditions.

4.7.4 Probing ES Products With Sera Obtained From Trial 2

The sera obtained from sequential and final bleeds of rats that had flukes and pathology in trial 2 (I_2) were used to probe the ES of D0, D14, D28, D42 and

Table 4.11 Apparent molecular weights of proteins in saline somatic extracts of flukes run under reducing and non-reducing conditions

Reducing			Non-reducing		
Age of flukes in days					
28	42	56	28	42	56
-	43	-	146	-	-
41	-	-	134	-	-
-	36-39	-	92	92	-
30-36	-	30	-	69	-
-	24	-	66-76	-	67
-	23	-	55	55	-
21	-	21	46	46	-
20	20	-	43	43	-
18	-	-	39	39	-
-	16	-	31-35	31-35	-
-	-	14	-	-	28-30
<14	-	<14	21	-	-
			17	-	-
			<14	-	-
			<14	-	-

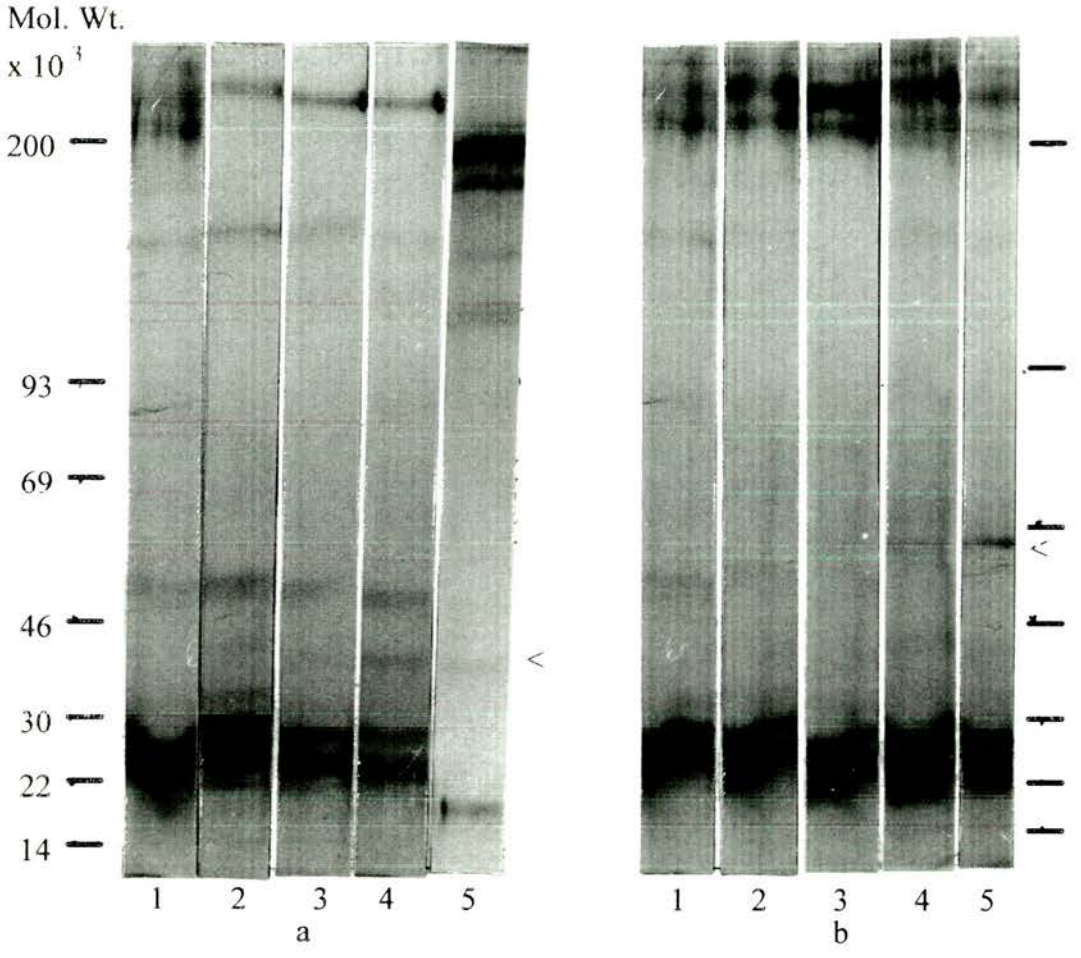


Figure 4.49.

Immune-coprecipitation of biosynthetically radio-labelled ES proteins of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 1. Gels were run under either reducing (a) or non-reducing (b) conditions.

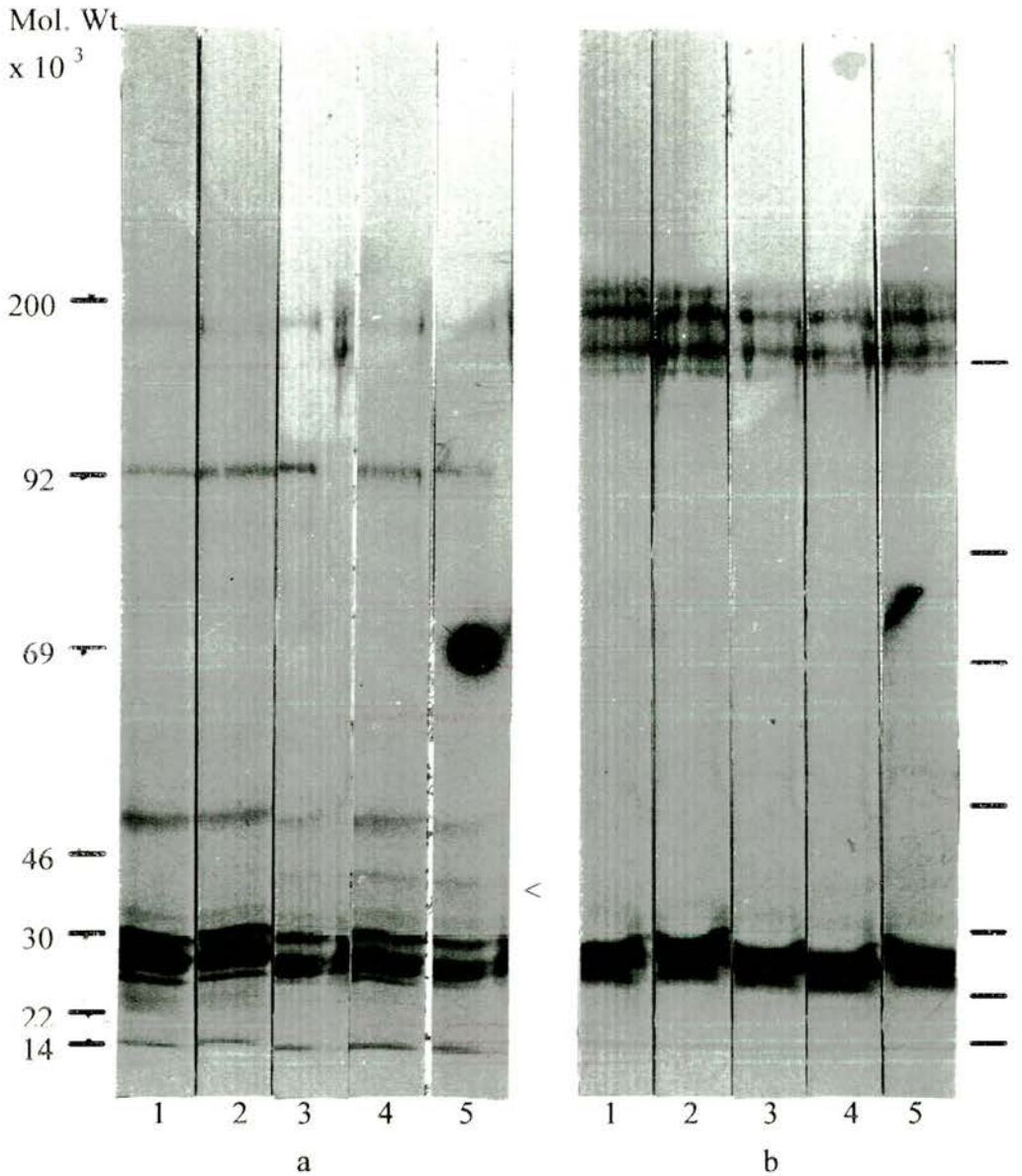


Figure 4.50.

Immune-coprecipitation of biosynthetically radio-labelled ES proteins of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with no flukes but pathology in trial 1. Gels were run under either reducing (a) or non-reducing (b) conditions.

adult flukes. In addition, sera from rats that had liver pathology but no flukes and those that had neither liver pathology nor flukes were used to probe ES_{14}.

4.7.4.1 Probing with sera from rats with flukes and pathology

D14 and D28 sera from these rats recognised an antigen with apparent weight of about 26 kDa in ES_0 run under reducing conditions. This antigen was hardly recognised by sera obtained at day 56. (Figure 4.51). The non-reduced form of this ES was not probed due to limited availability of the ES.

D0 and D14 sera did not recognise any antigens in ES_{14}. A major antigen of about 35 kDa was recognised in the reduced form of this ES by D28, D42, and D56 sera (I_2). This antigen was not recognised in the non-reduced form of the ES (Figure 4.52).

An antigen of about 35 kDa was recognised in the reduced form of ES_{28} by D14, D28, D42 and D56 sera. These sera did not recognise any antigens in the non-reduced form of this ES (Figure 4.53).

D0 sera did not recognise any antigens in ES_{42} but D14 sera detected an antigen of about 38 kDa in this ES run under reducing conditions. This antigen was also recognised by D28, D42 and D56 sera. The antigen was recognised as a major antigen by these sera. (Figure 4.54).

An antigen with apparent weight of about 38 kDa was recognised in the reduced form of ES_A by D14, D28, D42 and D56 sera. The recognition of this antigen was very faint. No antigens were detected in the non-reduced form of this ES. (Figure 4.55).

4.7.4.2 Probing with sera from rats with pathology but no flukes

D0, D14 and D28 sera from these rats did not specifically recognise any antigens in ES_{14} run under reducing conditions that were not recognised by D0 serum but D42 sera very faintly recognised an antigen of about 35 kDa in the ES. Sera obtained at day 56 of infection also faintly recognised this antigen (Figure 4.56).

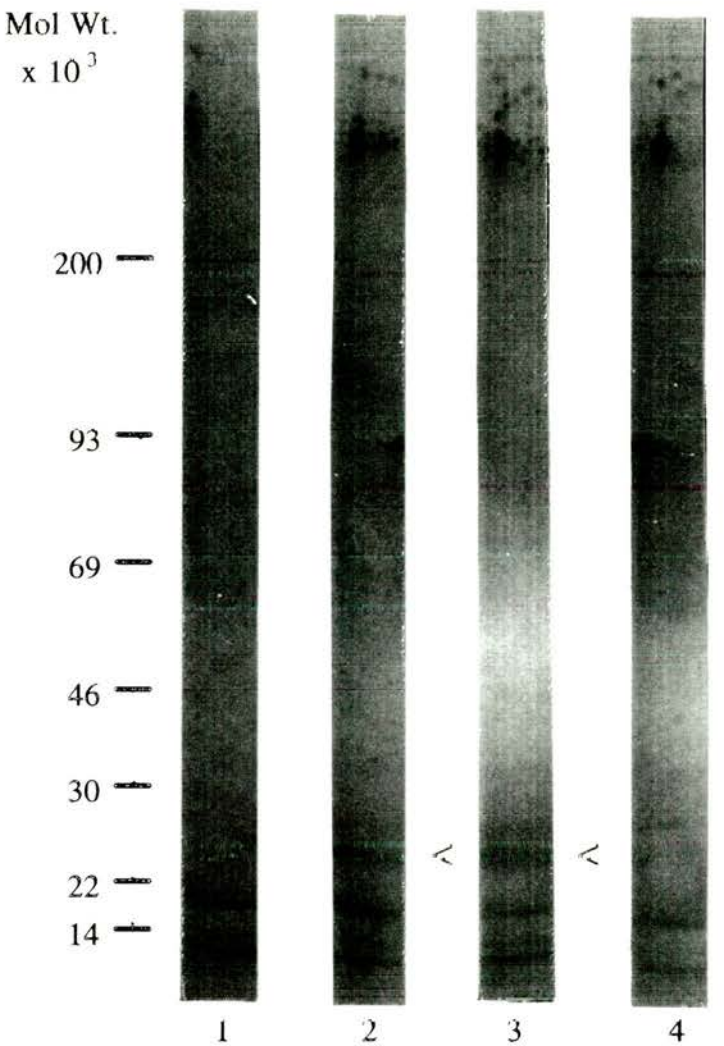


Figure 4.51.

Biosynthetically radio-labelled ES proteins of D0 *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3) and D56 (track 4) sera from rats with flukes and pathology in trial 2. Gel was run under reducing conditions.

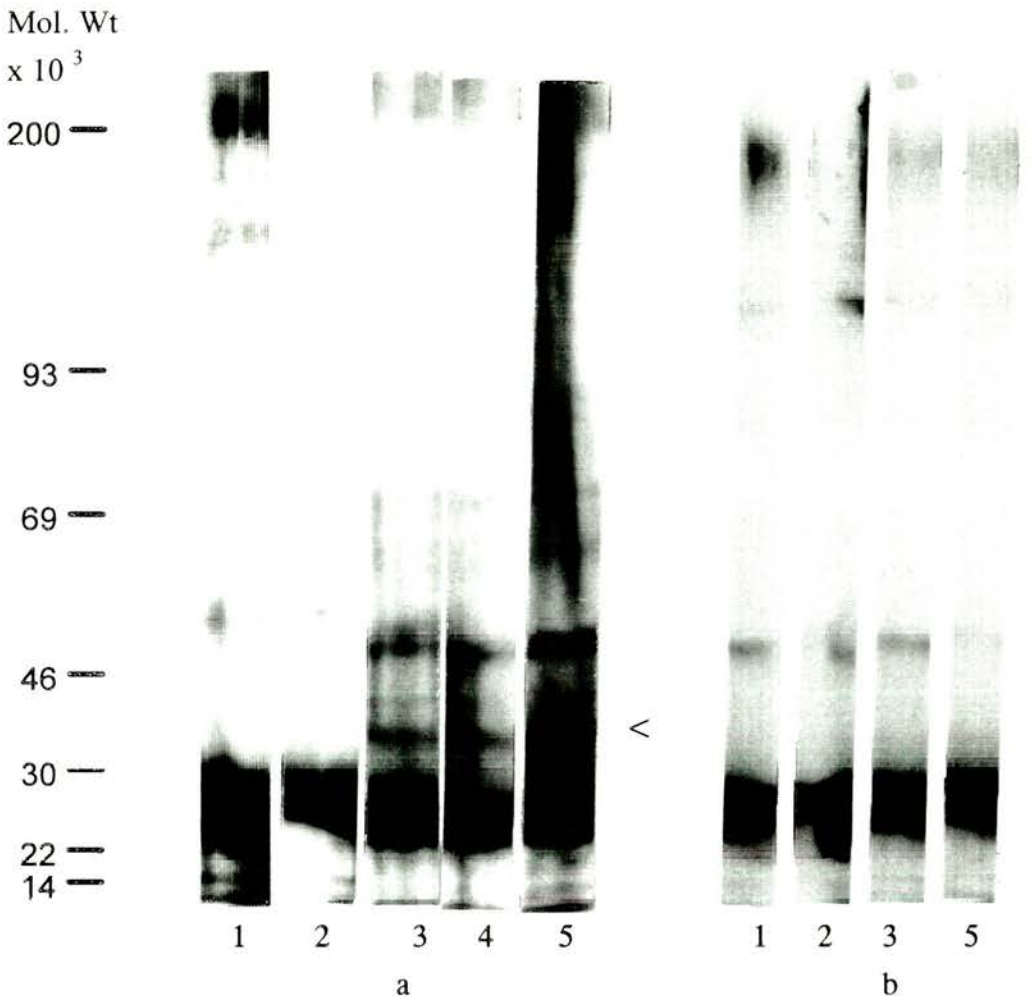


Figure 4.52.

Biosynthetically radio-labelled ES proteins of D14 *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

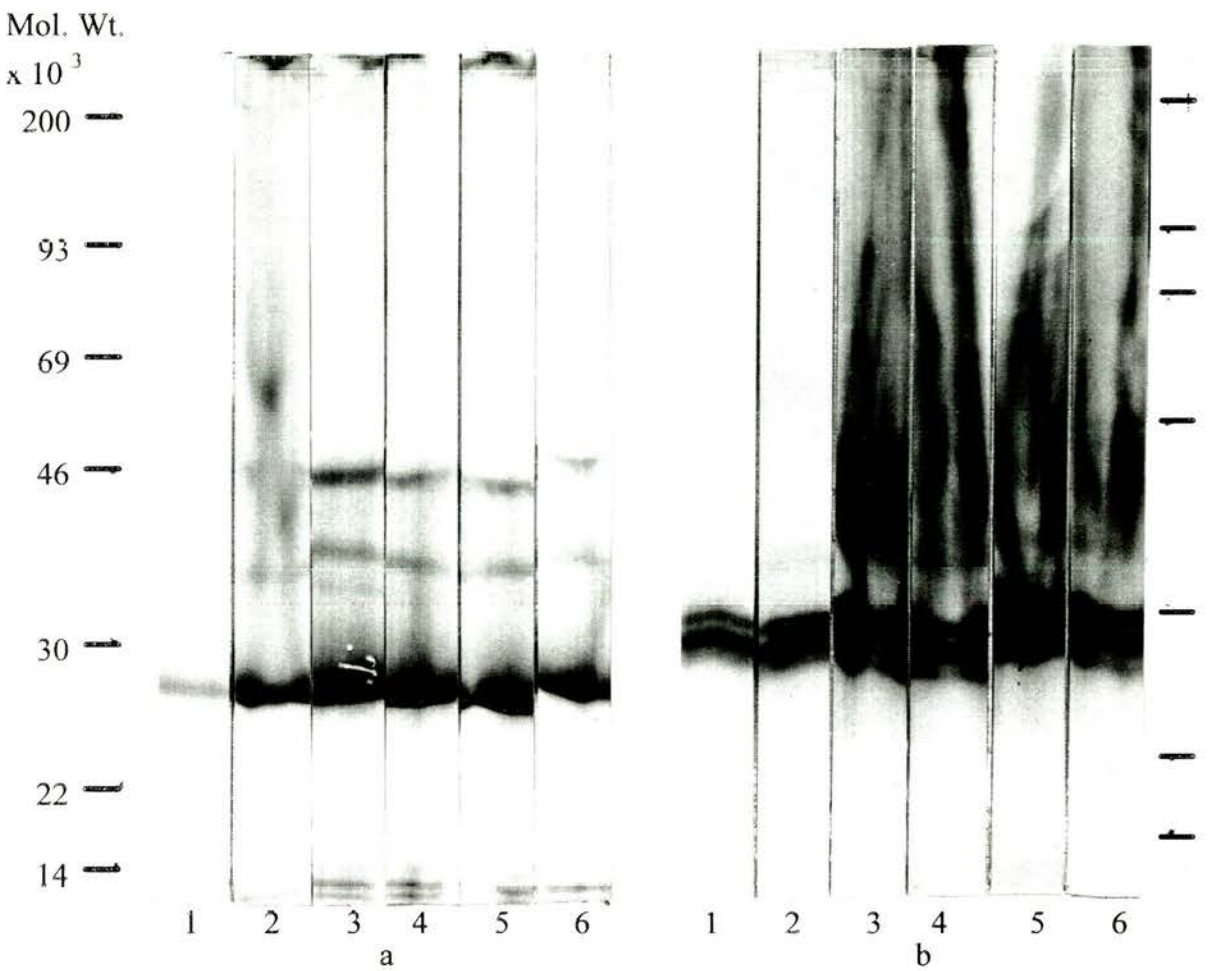


Figure 53.

Biosynthetically radiolabelled ES proteins of D28 *F. hepatica* probed with PBS (track 1) and D0 (track 2), D14 (track 3), D28 (track 4), D42 (track 5) and D56 (track 6) sera from rats with flukes and pathology in trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions

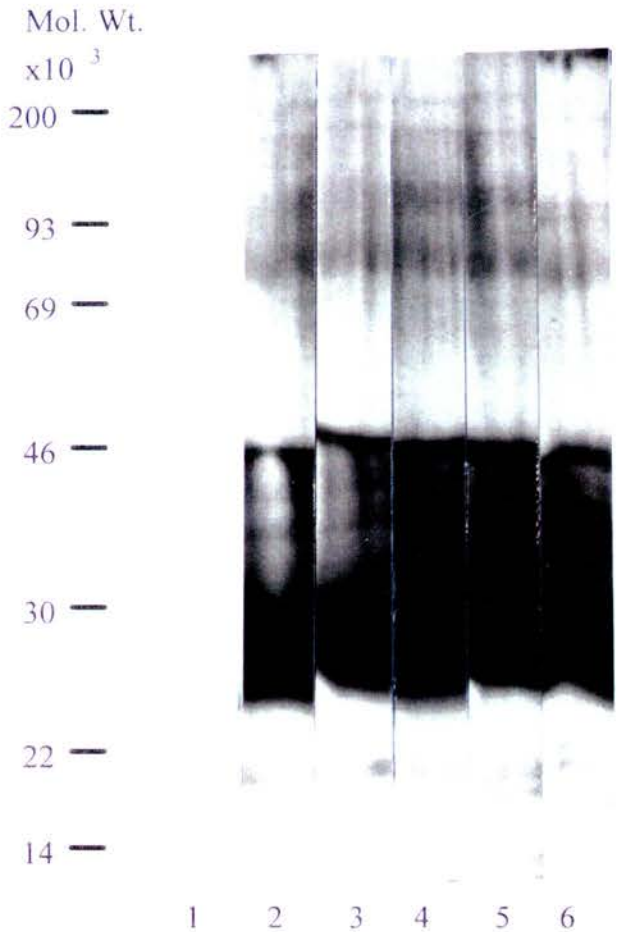


Figure 4.54.

Immune coprecipitation of biosynthetically radio-labelled ES proteins of D42 *F. hepatica* probed with PBS (track 1), and D0 (track 2), D14 (track 3), D28 (track 4), D42 (track 5) and D56 (track 6) sera from rats with flukes and pathology in trial 2. Gel was run under reducing conditions.

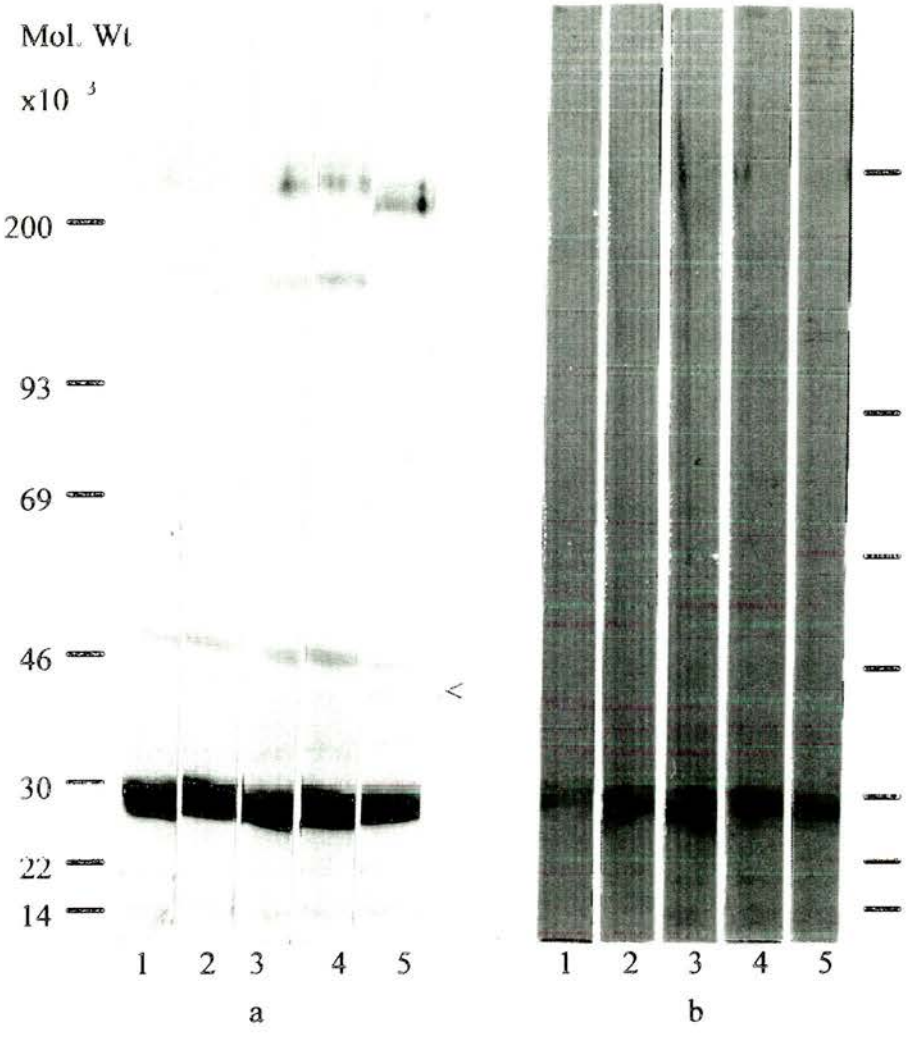


Figure 4.55.

Immune co-precipitation of biosynthetically radio-labelled ES proteins of adult *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with flukes and pathology in trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

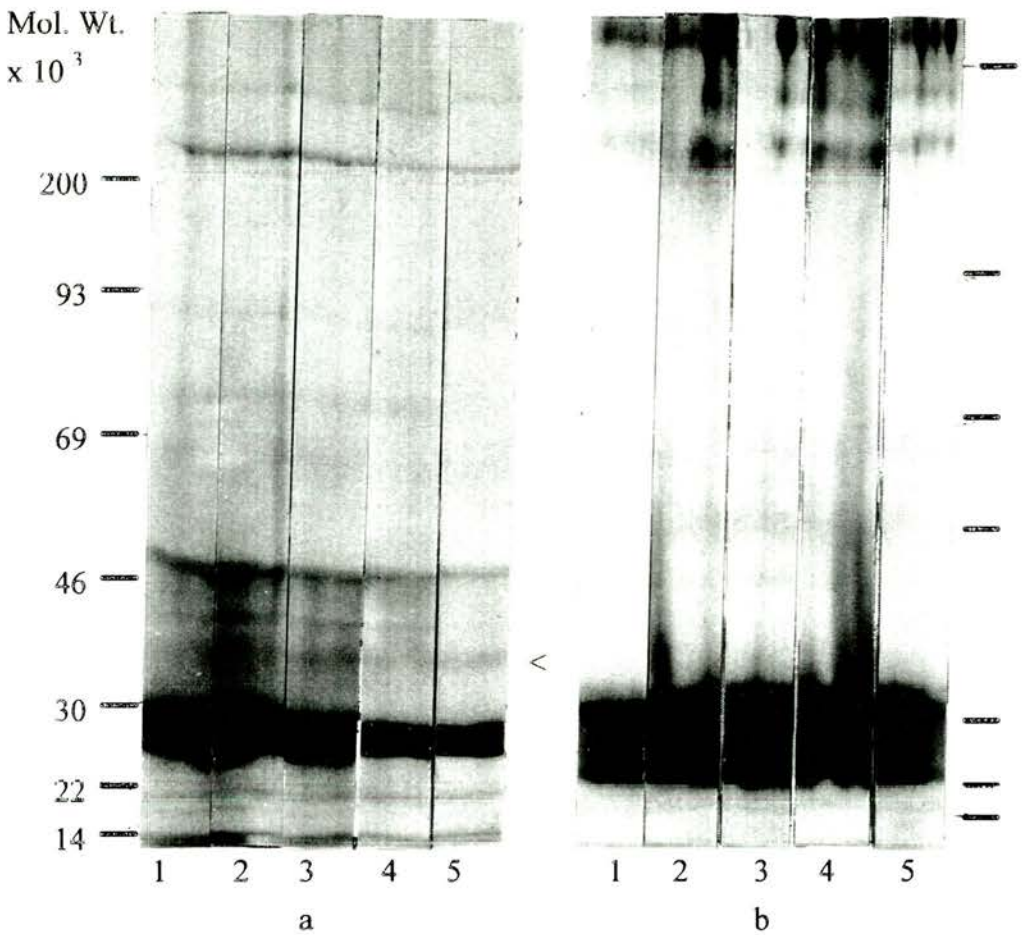


Figure 4.56.

Immune-coprecipitation of biosynthetically radio-labelled ES proteins of D14 *F. hepatica* probed with D0 (track 1), D14 (track 2), D28 (track 3), D42 (track 4) and D56 (track 5) sera from rats with no flukes but pathology in trial 2. Gels were run under either reducing (a) or non-reducing (b) conditions.

4.7.4.3 Probing with sera from rats with neither fluke nor pathology

Neither preinfection nor any sequential post-infection sera obtained from rats with neither flukes nor pathology in trial 2 recognised any antigens in ES_{14} run under reducing or non-reducing conditions.

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

The rat (Thorpe, 1965; Hayes *et al.*, 1972, 1974c) and cattle (Ross, 1967; Doyle, 1971) unlike sheep (Sinclair, 1962; Boray, 1967; Rushton, 1977), are known to be capable of developing protective immunity against *F. hepatica* following a primary infection. The ability of mice (Lang, 1967; 1968; Harness, *et al.*, 1977a; b) and rabbits (Healy, 1955; Ross, 1966; Kendall *et al.*, 1967; Kendall and Sinclair, 1971; Fortmeyer, 1973; Bolbol, 1975) to develop protective immunity appears controversial. In the rat this immunity is effective against oral challenge with metacercariae or parenteral challenge with newly excysted (Goose and MacGregor, 1973; Doy *et al.*, 1978), juvenile or adult flukes (Goose and MacGregor, 1973; Hughes, Anderson and Harness, 1976). Sera from infected rats and cattle is able to confer resistance against challenge in naive recipients, indicating the involvement, direct or otherwise, of antibody (Rajasekariah and Howell, 1979; Pfister *et al.*, 1984/85; Corba *et al.*, 1971; Haroun *et al.*, 1981).

The rat is often used, as in this study, as a laboratory model for cattle and for studying host-parasite interactions in fasciolosis due to its similarity to the cattle.

Interestingly, sera from hosts such as rabbits and sheep which are known to be incapable of mounting resistance after a primary exposure have also been shown to be capable of conferring protection when transferred to naive heterologous recipients (Haroun, 1979, Haroun *et al.*, 1981).

The successful establishment of the fluke infection supports earlier views (Lammler, 1959; Thorpe, 1965) on the suitability of outbred albino rats, the strain selected for this study, as laboratory hosts for *F. hepatica.*

In this study rats were infected at 5-6 weeks of age, a time at which they are fully susceptible to infection with *F. hepatica* (Goose and MacGregor, 1974; Rajasekariah and Howell, 1977). A slight unintentional mortality occurred in rats given an oral dose of 10-20 metacercariae, however, this dose rate was generally suitable for the production of a chronic infection.

Variation was observed in the percentage establishment of flukes in the three trials. Rats in the first trial each received a dose of 10 metacercariae while rats in trials 2 and 3 each received a dose of 20 metacercariae but the percentage of establishment varied widely with more flukes becoming established in primary infection for trials 1 and 3 than trial 2. It is therefore possible that the metacercariae used in the two former trials were more infective than those used in trial 2.

The metacercariae used in the three trials were obtained from different sources and this might be a source of variation (Dawes, 1962). Even batches of metacercariae of similar morphology derived from the same source on different occasions have been shown to differ in infectivity (Thorpe, 1965). Individual differences in the immune responsiveness of the rats to *F. hepatica* could well be expected in outbred rats and may also have been responsible for such variation (Thorpe, 1965; Pfister *et al.*, 1984\85) but variation may be expected to be evened out when using groups of experimental hosts. Urquhart (1954), after giving a standard dose of metacercariae observed differences in the number of flukes developing in rabbits but attributed this to differences in technique for dosing individual animals. In the present trials, the technique of dosing could not be ruled out as a factor since rats infected by direct stomach tubing in trial 3 had, on the average, more flukes per rat than rats infected by oesophageal intubation in trial 1.

It was interesting that in some rats no flukes were recovered but liver pathology was evident. In such rats the infection must have established initially as evidenced by the immune response measured by ELISA but was thereafter eliminated. The mechanism for this elimination is not exactly known. In those rats with neither flukes nor pathology, it is probable that the infection was eliminated at a very early stage. These two groups might be inherent high responder groups of rats.

The distribution pattern of the fluke recovered at post mortem in trials 1 and 2 were similar and both appeared to have a Poisson distribution. Thus the majority

of infected rats had only a light infection, a pattern of distribution similar to what might be expected in a natural infection. Even though the pattern of distribution was slightly different in trial 3 in that the rats generally had a heavier fluke burden, the majority of the infected rats still haboured comparatively low burdens.

The degree of protection afforded by the passive transfer of antiserum was poorer in trial 1 than trials 2 and 3, in which the degree of protection was statistically significant. However, the protection conferred by immune serum in all the three trials was comparable to that reported by previous workers (Hayes *et al.*, 1974a; Haroun *et al.*, 1981). The results of the present trials as well as those of previous workers point to the fact that antibody does not confer complete (100%) protection in fasciolosis. This might be expected since natural infection does not stimulate sterile immunity (Hayes *et al.*, 1974a; Hughes, Anderson and Harness, 1976; Boray, 1967). Moreover, cellular responses are known to play a role in immunity to fasciolosis (Lang *et al.*, 1967; Corba *et al.*, 1971; Rajasekariah and Howell, 1979; Armour and Dargie, 1974). The inability of antibody as well as active immunization with synthesized or purified antigens (Balloul, Grzych, Pierce and Capron, 1987; Wolowczuk, Auriault, Grass-Masse, Vendeville, Balloul, Tartar and Capron, 1989; Wolowczuk, Auriault, Bossus, Boulanger, Grass-Masse, Mazingue, Pierce, Grezel, Reid, Tartar and Capron, 1991) to confer complete protection upon recipients has also been observed in *S. mansoni*, a closely related trematode. Non-the-less, the present study confirms that antibody plays a significant role in protection against infection with *Fasciola* spp.

It is difficult to explain why a significant protection was not achieved in the first trial. The fact that serum used for this trial was obtained from rats that had a primary infection of 10 metacercariae (compared to dose of 20 received by rats used to raise antiserum in trials 2 and 3) might have contributed to this. However, the aggregate number of flukes of all rats from which the antiserum (I_1) was pooled was 67. This is not markedly different from the aggregate number of flukes (69) of all

rats used to produce antiserum (I_2) transferred in the second passive protection trial. The number of fluke per rat were similar, at 2.23 and 2.225 respectively. Rajasekariah and Howell (1979) reported that serum from rats that had experienced a primary infection of 5 metacercariae conferred a significant protection against oral challenge in homologous recipients. Thus a dose of 10-20 metacercariae used in this trial would have been expected to be enough. Although all metacercariae used were apparently viable as assessed visually, it is possible that they varied in infectivity and this might affect the number of flukes recovered at post mortem examination. It is noteworthy that the design of the passive protection trials does not represent what happens in the natural situation. Under natural conditions the sensitized intestine of infected animals acts as the initial barrier to the invading newly excysted flukes (Hayes and Mitrovic, 1977; Rajasekariah and Howell, 1977), apart from the fact that all tissues of the host are permeated by immunoglobulin. It is quite probable that in the present trials the immunoglobulin in the injected antiserum might not have permeated the intestinal and other tissues well enough before the penetration of intestinal walls by the invading flukes. If the antiserum had been injected about 24h before challenge there might have been better protection than was observed.

Rats that received normal rat serum before infection with metacercariae in the three trials consistently had a lower fluke burden than challenge control rats. This apparent 'protection' afforded by normal rat serum was also noted by Hayes *et al.*, (1974b); Haroun *et al.*,(1981); Mitchell *et al.*,(1981) and Chapman and Mitchell (1982a) and emphasises the importance of including this control in such studies. The protection is generally considered to be due to non-specific deleterious effects of the serum on the fragile newly excysted flukes in the abdominal cavity (Mitchell *et al.*, 1981).

Rats that received antiserum from infected rats from trials 2 and 3 habouring flukes and liver pathology at post mortem had less liver damage than the normal serum or challenge control rats. This is a good indication that in the first group of

rats most of the challenge flukes did not reach the liver, but must have been destroyed in the abdominal cavity. A similar observation was made by Rajasekariah and Howell (1977). Haroun *et al.* (1981) found that the serum glutamic dehydrogenase levels in rats that received protective antiserum at challenge were lower than those of normal serum and challenge control rats, which supports the present observation relating to liver damage. The relative failure of passive protection in trial 1 was reflected in the comparatively greater amount of liver pathology in rats that received antiserum to *F. hepatica* in trial 1 as compared to trials 2 and 3.

The sizes of flukes recovered from rats that received antiserum in the three trials were similar to those recovered from normal rat serum and challenge control rats. This confirms the results of previous workers and suggests that immune serum has an "all or non effect" and those flukes that survive the effect of the immune serum were able to develop normally (Haroun, 1979).

The pattern of antibody response (as monitored by ELISA using ES and somatic extracts of adult fluke as antigens) of rats following a primary infection with *F. hepatica* was similar in the three trials. The early antibody response observed in infected rats by two weeks post infection against both parasite extracts used suggests the existence of common antigens between the juvenile and adult stages. By 14 days post infection, when serum was first collected the young migrating juvenile flukes are already in the liver parenchyma. The steady increase in antibody response suggests that the growth of the fluke in the liver parenchyma is associated with continued stimulation of the host's immune responses. This is probably because as the fluke grows its ability to produce metabolic products increases. The migration of the young adult flukes into the bile ducts after D42 of infection coincided with a decline in serum antibody. A similar pattern has been described in rabbits (Lehner and Sewell, 1980) and rats (Oldham, 1985; Poitou,

et al., 1992) infected with *F. hepatica*. However, the latter authors obtained a second antibody peak at 10 weeks after infection.

Rats given immune serum in trial 1 had an elevated antibody level on day 14 (blood samples were not taken on days 1 and 3 from these rats). This initial elevation was probably in part the result of the injected antiserum but, as the rats were not significantly protected this initial elevation will have been maintained by the response stimulated by the flukes resulting from the infection. In contrast, in trials 2 and 3 this group of rats showed elevated antibody levels on days 1 and 3 but, as most of the flukes appear to have been killed by the injected antiserum, the antibody level declined thereafter. The slight elevation in antibody response seen in trial 3 may be attributed to the fact that most of these rats had flukes even though there was an overall significant reduction in their fluke burden.

The pattern of eosinophilia observed in rats during primary infection in the present study was similar to available reports (see literature review). As total cell counts were not done, it is impossible to know if this was an absolute or relative eosinophilia. Non-the-less, the eosinophilia increased relative to preinfection levels in the passive protection groups of rats at D28 post infection and rats that received antiserum had lower eosinophil levels than rats given normal rat serum or challenge control rats.

From the report of Milbourne and Howell (1990) that rats and mice infected with *F. hepatica* or injected with ES products of adult fluke showed a rapid increase in peripheral eosinophils, it could be suggested that the eosinophilia observed in the present study was at least in part a response to ES products from the flukes. Thus, in rats that received antiserum and which had significantly fewer flukes than the normal serum and challenge control rats, there might have been less ES from the flukes and hence, a lesser eosinophilia.

Immunochemical Studies

In the immunochemical studies the total ES products of various ages of flukes as well as detergent extracts of adult fluke were analysed by western blotting. This is a powerful analytical tool that has been employed in studying various parasites (Harrison, Parkhouse and Sewell, 1984; Gottstein, 1985; Janssen, Wit, Rycke, De-Wit and De-Rycke, 1990; Homan, Derksen, Knapen and Van-Knapen, 1992). A disadvantage of this technique is the possibility of low molecular weight proteins failing to bind or binding weakly to the support matrix (Hames and Rickwood, 1990).

Biosynthetic labelling, which was also employed in the present study, has the advantage that it affords the opportunity to study ES components which are actively excreted and or secreted by a parasite, thus excluding waste and breakdown products that could be present in total protein recovered from parasite cultures (Joshua *et al.*, 1988). Biosynthetic labelling has been employed by many investigators (Parkhouse and Clark, 1983; Harnett, Meghji, Worms and Parkhouse, 1986; Sugane, Howell and Nichola, 1985; Wiest, Tisdale, Roberts, Rosenberry, Mahmoud, and Tartakoff, 1988). The main disadvantage of this technique is the fact that some of the proteins being synthesized by a parasite might be utilising amino acids, other than the one being used in the labelling, as precursors. In addition, the extent of incorporation is influenced by factors such as the amount of a particular amino acid in a protein molecule (Colligan, Kruisbeek, Margulies, Shevach and Strober, 1992) and the rate of metabolism of the parasite at any particular point in its development (Harrison, personal communication).

Analysis of Fluke Extracts

PBS-PI-NP40 extracts of biosynthetically radio-labelled D28, D42 and adult flukes as well as PBS-PI and surface detergent strips of adult flukes were studied to a limited extent.

The somatic (PBS-PI-NP40) extracts of ^{35}S-methionine labelled D28, D42 and adult flukes did not contain many components. Compared to the extracts of D28 and D42, the adult fluke extract contained even fewer components. This may simply have been because the autoradiographs, especially that of the adult fluke extract, were not allowed to develop for a sufficiently long time.

The NoG extract apparently contained more components than the CTAB extract. This seems to suggest that the two detergent extracts vary in the degree to which they stripped the surface coats of the flukes. The former detergent has been shown to be efficient for stripping the surface coats of *Nematospiroides dubius* (Pritchard, Crawford, Duce and Behnke, 1985).

Apart from a singlet of about 25 kDa which appeared to be common to the two extracts, there were no other similarities in antigens recognised by antiserum in the extracts.

In western blot studies antigens of about <15, 25, 38, 48 and 62 kDa were recognised in the NoG extract by D14 and subsequent sera from rats with flukes and pathology in trial 2. This indicates that these components may be common to the juvenile and adult flukes. These as well as other antigens (25 and 48 kDa) identified in CTAB may be useful in early diagnosis of infection. Since the surface compartment is a target for immune attack some of the components recognised in these particular extracts could be of significance in protection.

Antigens recognised in the somatic extract are unlikely to be of diagnostic or protective significance. This is because the host is only exposed to components of this compartment after the death and disintegration of the parasite. However, exposure to these components at this time may provoke a pathological reaction (Parkhouse and Harrison, 1989).

Fewer antigenic components were detected in the PBS-PI extract examined in this present study than were reported by Sloan *et al.* (1991) who probed homogenates of adult flukes with sera from infected rats as well as monoclonal

antibodies produced from rats that had been infected with *F. hepatica*. These authors had made their adult fluke homogenates in SDS-buffer without any protease inhibitors.

The 53kDa antigen recognised in the present study may be similar to a 53 kDa component reported by Keegan and Trudgett (1992) who used sera from infected rats to probe western blots of adult fluke homogenate. However, several other components (78, 95, 133 and 193 kDa) reported by these authors were not recognised in the present study. Similarly, antigens of about <15, 22, 25, and 43 kDa recognised in the present study were not detected by the authors. These authors did not state the method used to prepare their homogenates. Secondly, the authors used sera diluted at 1:50 while in the present study sera was used at a dilution of 1:200.

The <15 kDa antigenic component detected in this study has about the same size as the <14 kDa component detected by sera from infected rabbits in Western blots of fractions (14-45 kDa) obtained from gel filtration of a homogenate of adult flukes (Santiago and Hillyer, 1986). Other antigenic components detected by rabbit antiserum were not detected in the present study, while components of 22 and 53 kDa detected in the present study were not detected by these authors. As discussed earlier, the rat, unlike the rabbit, is similar to cattle (the appropriate host) in its response to infection with *Fasciola* spp. The observed differences in antibody response may well be a reflection of differences in antibody response of an appropriate (rat) and inappropriate (rabbit) host. Such differences have been demonstrated in infection of mice and cattle with *Taenia saginata* metacestodes (Harrison, Parkhouse and Sewell, 1984). These differences may also have resulted from the fact that these authors prepared their homogenates in PBS without the inclusion of protease inhibitors.

Analysis of ES Products of Flukes Maintained in Culture

The silver stained protein profile of D0 and D1 ES were relatively simple in contrast to those of D14 and adult flukes, which contained numerous components. This could either mean that D0 and D1 flukes synthesized very few components over the 24h culture period or that they produced much less material than the larger older flukes. The simple profile of D0 and D1 fluke might also be related to the low protein concentration of their ES products, which meant that about 15 times less protein (0.03 ng and 0.08 ng of D0 and D1 ES/mm^2 of gel as opposed to 0.5 ng of D14 and adult ES/mm^2 of gel) was loaded into the gels and minor components could not be detected.

Although the complete culture medium was run at the same concentration as the D14 and adult ES, and about 15 times more protein than the D0 and D1 ES, it only revealed one component of about 67 kDa in the reduced state. This component is likely to be bovine serum albumin from the foetal calf serum used in the medium. This component might be or be part of the 63-77, 54-76, 67 and 60-75 kDa components found in the reduced D0, D1, D14 and adult fluke ES respectively. Similarly, the component with apparent weight of 69 kDa in the non-reduced form of medium is likely to be part of the components of about 56-66 kDa in D0 and D1 ES and components with apparent weight of 62 kDa and 69 kDa in D14 and adult fluke ES respectively. It is apparent, therefore, that the other components detected in ES of various ages of flukes were of parasite origin.

The similarity between ES of D0 and D1 flukes on one hand and D14 and adult flukes on the other hand may be related to maturation. It might also mirror the similarity of the secretory granules of flukes of these two ages. Newly excysted juveniles and young invading flukes (D1) are known to possess T0 granules which are secreted onto the tegument, while the T1 granules are responsible for secretions in the young and adult flukes (Hanna, 1980b).

A comparison of the profiles of the biosynthetically radio-labelled ES proteins with those of silver stained ES reveals that the former generally contained fewer components than the latter, especially so for D14 and adult flukes. This may be because biosynthetically radio-labelled components represent only those components being actively synthesized during the culture period while the total ES obtained from culture also includes parasite breakdown and waste products. As already discussed, the amount of biosynthetically radio-labelled components revealed is also influenced by the rate of metabolism of the flukes as well as the composition of the proteins.

The major protein detected in the non-reduced ES of D0 fluke is likely to be passively labelled bovine serum albumin. Such passively labelled bovine serum albumin was observed by Parkhouse, Clark, Maizels and Denham (1985) in biosynthetic labelling of *Brugia pahangi*. As the passively labelled molecule was precipitated by rat-anti-bovine serum albumin, the authors suggested that ^{35}S-methionine had complexed via sulphydryl linkage to the BSA in FCS during culture period. This phenomenon does not seem to occur consistently. It was not observed in biosynthetic labelling of *T. spiralis* (Parkhouse and Ortega-Pierres, 1984) but has been observed in *Litomosomoisoides carinii* (Harnett, Meghji, Worms and Parkhouse, 1986) and in *T. saginata* (Joshua *et al.*, 1988). In this study the passive labelling did not occur during biosynthetic labelling of D14 and D28 flukes. As yet there is no clear explanation for the occurence of this phenomenon.

The differences in composition of biosynthetically radio-labelled ES of various ages of flukes might be a reflection of the differences in functions of these various ages.

The biosynthetically radio-labelled D0 ES contained relatively less components than its unlabelled form and also less components than the biosynthetically radio-labelled D1 ES. This may mean that the D1 flukes are

metabolically more active than the D0 or that the D0 has preformed pools of most of the proteins it needs.

The main role of the D0 fluke is the penetration of the intestinal wall and entry into the abdominal cavity. Thus, it is possible that the components it excretes or synthesized might be important for this activity. Also, at this stage of development the parasite has to radically change its environment from the gut to the abdominal cavity.

The D1 fluke stays in the abdominal cavity and the developing flukes penetrate the liver by D2 or D3 (Dawes, 1964). Thus biosynthetically radio-labelled components specific to D1 (36, 78, 82, 103, 155 and 168kDa in reduced form and 28, 40, 55 and >200 in the non-reduced form) could have enzymatic functions necessary to digest the liver tissue. Enzymatic functions have been detected in the ES of many heliminth parasites (Chapbell and Dresden, 1986; McKerrow, Pino-Heiss, Lindquist and Werb, 1985; Hotez and Cerami, 1983; Hotez, Le Trang, McKerrow and Cerami, 1985; Petralanda, Yarzabal and Piessens, 1986; Lindquist, Senft, Petit and McKerrow, 1986; Robertson, Bianco, McKerrow and Maizels, 1989).

There were many apparent similarities in the biosynthetically radio-labelled ES of D14, D28 and D42 flukes. In reduced form new components of about 39kDa (40 kDa in ES of D42) and 26-30 kDa were common to the three stages while new components of 36 and 46 kDa were detected in the non-reduced ES. These three ages of flukes are all parenchymal flukes, and apparently they are the stage most responsible for the host's immune response. It is therefore also probable that components specific to the ES of these flukes could play an important role in the evasion of the host's immune response. These components could be suppressive to lymphocyte's functions or toxic to lymphocytes as has been demonstrated in *Taenia taeniaformis* and adult *F. hepatica* respectively (Burger *et al.*, 1986; Goose, 1978). These ES components could also cause the alteration of functions of host

macrophages, mast cells and granulocytes as been shown in infections with *S. mansoni* (Butterworth, Remold, Houba, David, Franks, David and Sturrock, 1977; Capron, Torpier and Capron, 1979; Mazingue, Camus, Dessaint, Capron and Capron, 1980). Since these flukes, like the younger ones, also feed on blood and liver tissue it is not unlikely that components in their ES might have proteolytic functions.

ES of D42 fluke contained a new component of about 119kDa. This stage of fluke must penetrate the bile ducts at about D50 (Dawes, 1964) and since this component appears to be specific to this stage, it might be relevant to this penetration. Similarly, the two new components (87 and 107 kDa) detected in ES of adult flukes might be useful for adaptation of the fluke in its new bile duct environment.

Western Blotting of ES Products

The ability of anti-*F. hepatica* antibodies to confer some degree of protection against challenge infection (see literature review) was confirmed in the passive protection trials described in this study. These antisera containing the protective antibodies and also antisera from rats in the passive protection trials were therefore used in western blotting studies to probe the ES of various ages of flukes in order to identify components that might have a role in protection or be of diagnostic significance.

No antigens were detected if the antiserum was used to probe the complete medium run under either reducing or non-reducing conditions. This clearly indicates that all the antigens detected in the various ES products were of parasite origin.

In this study, an antigen of about 191kDa was recognised in the ES products of D0 and D1 flukes. Although this antigen was not recognised by sera collected earlier than D42, the 191kDa component must obviously have been present in the

flukes before D42 but antibodies could not be detected due to assay sensitivity and the small amounts of parasite ES obtained from D0 and D1 flukes. The fact that the antigen was recognised by D42 and D56 sera suggests that the antibody to this antigen persists in the serum for a long time. Earlier workers (Bennett, 1978; Sandeman and Howell, 1981; Yoshihara *et al.*, 1981) demonstrated that metacercarial extracts (that is, D0) and newly excysted juvenile (D1) flukes formed precipitin lines in immunodiffusion reactions with antisera raised in rabbits against a soluble extract of adult fluke, and antisera from infected rats and rabbits respectively. The present study has extended this knowledge by characterizing the antigen (191 kDa) that might be involved in this reaction at the molecular level. Being produced by both the infective (D0) and invasive (D1) stages of *F. hepatica*, this antigen revealed in the present study is likely to be useful in immunodiagnostic assays designed to detect antiparasite antibody in infected animals especially since the antibody persists in the serum until the flukes are mature. In addition, as D0 and D1 flukes are those that are first exposed to the host's immune system, antigens recognised in their ES might have a protective significance for the invading flukes of a secondary challenge. That this antigen might be involved in protection is supported by comparing the pattern of its recognition by pooled sera from rats injected with antiserum in passive protection trial 2 with that of pooled serum from rats in this group which had neither flukes nor pathology at post mortem. The fact that in the latter group of rats this antigen was strongly recognised by pooled D1 and D3 sera after which the recognition decreased with time suggests the involvement of antibodies to this antigen in either direct antibody-mediated or eosinophil-mediated destruction or mediating eosinophil destruction of invading juvenile flukes. The later mechanism has been hypothesized for the destruction of larvae of *Toxocara canis* (Badley, Grieve, Rockey and Glickman, 1987).

Similarly, the 25 kDa doublet recognised in the ES of D14 flukes might be useful in host protection. This is especially so considering the fact that this antigen

was recognised by protective antiserum and that a significant protection against oral (Lang, 1974) and intraperitoneal (Lang and Dronen, 1972) challenge have been obtained in mice implanted with this age of fluke prior to challenge. This antigen has the same apparent weight as the 25 kDa singlet detected in the saline and detergent extracts of adult flukes, but it might not be the same as these singlets. It is apparent from the result of the second passive protection trial that the antibodies produced against the 25 kDa doublet might have been involved in the destruction of the juvenile flukes. Thus the antigen was strongly recognised by pooled D1, D3 as well as D42 and D56 sera from rats injected with antiserum. By contrast, only pooled D1 and D3 sera from rats injected with antiserum but which had no flukes and pathology at post mortem recognised this antigen. This implies that this component might be present in the ES or surface compartment of invasive flukes so that the antibodies which were produced by the host against an analogous component in D14 ES was able to kill or mediate the killing of the juveniles. This argument seems to be supported by the fact that immuno-coprecipitation of biosynthetically radio-labelled ES of D0 fluke detected an antigen of about 26 kDa, implying that this component is actively secreted by D0 flukes.

A doublet of about 25 kDa was recognised in the reduced form of adult ES. This doublet might be analogous to the 25 kDa doublet in D14 ES. It might likely be the reduced form of one of the two antigens (27 and 26 kDa) detected in the non-reduced form of the ES. These antigens were recognised by sera from rats used to raise antiserum in all three trials. The possible involvement of these components in the destruction direct or otherwise of invading flukes is suggested by the result of the second passive protection trial as discussed above. The fact that these components were apparently more strongly recognised by antiserum I_2 (compared to their recognition by antisera I_1 and I_3) might probably explain why antiserum I_2 conferred the highest level of protection and this in turn argues for the possible involvement of these antigens in promoting a protective response in the host.

The spectacular pattern of antigen recognition by sera from rats used in passive protection trial 2 was not obtained in the two other passive protection trials. In the first passive protection trial rats were not significantly protected and hence there was no noticeable decrease in recognition of the antigens. This means that before the injected antibody was cleared from circulation the flukes from the challenge infection had already started stimulating the production of antibody by the rats. Of the rats that received antiserum in passive protection trial 3 only two had no flukes at post mortem but they did have liver pathology. It is probable that in these two rats the challenge flukes were not completely eliminated by the antiserum in the abdominal cavity but were probably destroyed by the rat's immune system during the course of infection, in which case the rats would have been exposed to products from the flukes for some time.

The 25kDa antigen identified in reduced form of adult ES in this study was of similar size to a 22.5 kDa antigen identified by Poitou *et al.* (1992) in western blots of adult fluke ES by sera collected from rats from week 2 of infection with *F. hepatica*. However, the authors also reported the identification of several other components (see literature review) which were not identified in the present study. These differences could be due to a variety of reasons. Firstly, the authors obtained their sera from rats that were infected at 17-18 weeks of age. Secondly, the flukes were cultured in ordinary saline whereas throughout this study we maintained flukes in complete culture medium, a far richer medium, in order to protect the flukes from tegumental disintegration caused by a hostile culture environment. Thirdly they used sera diluted at 1:5 in skimmed milk, unlike in the present study when serum was diluted at 1:200. Unfortunately, the authors did not support their result with pictures of the western blots.

The 25 kDa antigen also falls within ranges (23-28 kDa) of proteins identified by Santiago *et al.* (1986) by sera of infected rabbits in western blots of adult fluke ES. However, other components reported by these authors (see literature

review) were not detected in the present study. These authors had collected ES from flukes that were maintained in ordinary PBS for 3h. In making this comparison it is assumed that these authors ran their proteins under reducing conditions since they did not state this in their papers.

The 25 kDa is also of similar size to components of 23 and 25-30 kDa detected by sera from infected rabbits in western blots of 'reactive' fractions obtained from gel filtration of adult fluke ES (Rivera-Marrero *et al.*, 1988). Again several other components (66, 95, 150-160) identified by these authors were not revealed in the present study. These differences might have arisen due to differences in host response. Apart from this, there were differences in culture conditions. These authors maintained their flukes in PBS and, in the case of Rivera-Marrero *et al.* (1988), with the addition of a protease inhibitor (PMSF) to the medium.

Of all the antigens recognised in western blots of adult fluke ES by monoclonal antibodies purified from ascites fluid of mice that had been immunized with ES of adult fluke (Solano *et al.*, 1991) only the 29 kDa appears close to the 25 kDa detected in the present study. Apart from differences in host response discussed earlier, the authors had maintained their flukes in Earle's salt solution without serum or any essential amino acids, a condition which may well have affected the composition of the ES.

Sera from rats with liver pathology but no flukes in rats that were infected for production of antiserum in trial 1 moderately recognised a doublet of about 19 kDa. This might be an important component worthy of further investigation since sera from rats with liver pathology and flukes in the three trials did not recognise such a component.

Sera from rats that had neither flukes nor pathology among rats infected for the production of antiserum in trials 1 and 2 did not detect any antigens in adult fluke ES. However, the ELISA response of this group suggests their exposure, at

least for a short time, to flukes. The lack of detection of antibody by these sera may therefore be related to assay sensitivity.

It is noteworthy that when sera from rats injected with antiserum in passive protection trial 2 was used to probe NoG surface extract, the 25 kDa singlet in the extract was recognised in a similar pattern as described for D14 and adult ES. Although it might not be the same protein as the 25 kDa doublet in D14 and adult ES, it does appear to be important in protection in view of the pattern of its recognition by sera from rats injected with antiserum but which had neither flukes nor pathology. It is probable that this antigen is present on the surface of D1 fluke, so that antibodies produced against the surface antigen of adult fluke was also effective against the surface of invading D1 fluke. Indeed, Lammas and Duffus (1985) identified a component of similar size (26 kDa) on surface of D1 flukes.

Immune-coprecipitation

An antigen of about 26 kDa was identified by D14 and D28 sera in biosynthetically radio-labelled D0 ES but not the equivalent of the 191 kDa identified in the unlabelled form of this ES. This might be an indication that while the former antigen utilises methionine as its precursor, the latter does not. It is possible that the 26 kDa component was present in the unlabelled ES of D0 and D1 flukes but in so small quantities as not to be detected by silver stained and western blot preparations. The fact that the 26 kDa antigen was identified by D14 sera means that it must have been produced by the flukes earlier than D14 of infection, and could therefore be useful for diagnosis. The detection of this antigen by D14 and D28 sera may suggest that the antibodies to this antigen persist for a long time in the circulation.

An antigen of about 38 kDa was recognised in biosynthetically radio-labelled ES of D14, D28 and D42 flukes by D14, D28, D42 and D56 sera from rats that were infected to produce the antiserum in trial 2. A component of about the

same molecular weight was recognised in the total biosynthetically radio-labelled D1 ES, suggesting that this component is produced by D1 fluke and as such might be of relevance to diagnosis and or protection. The fact that this 38 kDa antigen was recognised by a protective antiserum may indicate that it plays some role in parasite protection, in which case antibodies against it may be useful in host protection. It is probable that this antigen may have shared epitopes with the 25 kDa doublet detected in western blots of D14 ES.

This result of this immune-coprecipitation is at variance with that of Irving and Howell (1982) who reported that antiserum from rabbits that had been vaccinated with soluble extracts of adult fluke recognised components of 24, 26 and 27 kDa in biosynthetically (^{35}S-methionine) labelled ES of 3-week-old *F. hepatica*. There could be many reasons for these variations. The authors obtained their ES from flukes that had been maintained for 7 days in culture. The antisera they used was not from an oral infection but had been produced by vaccination and they were using a different host from that used in the present study.

An antigen of about 38 kDa was faintly recognised in biosynthetically radio-labelled adult fluke ES by sera obtained from rats used to produce antiserum in trials 1 and 2. A component of same size was detected in the biosynthetically radio-labelled D1 ES, suggesting that this component was produced by D1 flukes and might therefore be a useful diagnostic component. Since this antigen was recognised by protective antiserum (I_2) it may be useful for protection. The fact that it was only faintly recognised might mean that this antigen is not abundant or immunodominant.

The 38 kDa antigen appears to be of similar size as one of the antigens (40 kDa) detected in immunoprecipitation of antiserum from infected rabbits with radio-labelled adult fluke ES (Santiago *et al.*, 1986). However, several other antigens (28, 33, 44 and 62 kDa) detected by these authors were not recognised in the present study. These differences might be due to differences in host response as well as differences in culture conditions used in the two investigations. These authors

maintained their flukes for 3h in PBS without addition of serum or any of the essential amino acids, both of which could affect the composition of ES (Lehner, 1977; Kwan-Lim, Gregory, Selkirk, Partono and Maizels, 1989).

The results of the present study also differs from that of Sexton *et al.* (1991) who identified a major antigen of about 30 kDa in biosynthetically radio-labelled adult fluke ES. Apart from the fact that these authors used antiserum from a different host (sheep), their flukes were maintained in serum-free MEM, a condition that again might affect the composition of the ES.

None of the antigens (12.5, 13.5, 14.4, 26.5, 27 and 70 kDa) recognised by immunoprecipitation of mRNA *in vitro* translation products of adult flukes by antisera produced in rabbits against homogenates of *F. hepatica* (Hillyer and Taylor, 1988) were detected in the present study. Again the difference may be due to differences in host response and the fact that the two studies were probing different parasite preparations.

The results of this immuno-coprecipitations suggest that most of the biosynthetically radio-labelled ES components were apparently non-immunogenic. These non-immunogenic components could be very useful in diagnostic assays designed to detect parasite products in the circulation (Joshua *et al.*, 1988) by using monoclonal antibodies directed against such components. (Maizels, Denham and Sutanto, 1985). Moreover, some of these components are specific to some ages of flukes and monoclonal antibodies produced against such components could be of use in epidemiological studies aimed at determining the temporal distribution of the disease. Very immunogenic components are not very suitable for such assays (Parkhouse, Almond, Cabrera and Harnett, 1987) as they form complexes with host antibody resulting in reduced assay sensitivity (Forsyth, Spark, Kazura, Brown, Peters, Heywood, Dissanayake and Mitchell, 1985).

Conclusions

This study concentrated on the molecular characterization of the excretory/secretory products of *F. hepatica* as it develops in the rat and to a lesser extent, somatic (saline) and detergent surface extracts of adult fluke. It did demonstrate that changes occur in the ES products of the fluke in the course of its development in the rat and that certain components are specific to some ages of flukes. The study demonstrated that there are differences in protein profiles of total ES products and total biosynthetically radio-labelled ES proteins of all ages of flukes examined.

It has extended the study of Yoshihara *et al.* (1981); Sandeman and Howell (1981) and Benette *et al.* (1982) who used immunodiffusion to study antigenicity of metacercarial extracts by characterizing at molecular level a 191kDa antigen involved in immune reactions with the host.

It has identified molecules that are apparently immunogenic in unlabelled D14 and adult fluke ES. The differences of this result with those of earlier workers highlighted two points. First, that the method of collection of the ES product could affect its composition and hence antigenicity. Second, that there is considerable variation in different host responses to fluke ES.

The study also identified immunogenic and non-immunogenic components in biosynthetically radio-labelled ES of invasive (D1), parenchymal (D14, D28 and D42) and adult fluke. It is suggested that the immunogenic components might be important in diagnosis and or protection while monoclonal antibodies produced against the non-immunogenic components might be important in diagnostic assays aimed at detecting parasite products.

Finally, the study characterized components that appear to be involved in antibody-mediated protection. This includes antigens of 191, a 25 kDa doublet, 26 and 27 kDa; in D1; D14 and adult ES respectively as well as a 25 kDa antigen in

NoG detergent surface extracts of adult flukes. It is suggested that these antigens play an important role in host-parasite relationship in fasciolosis.

Suggested Studies

Only a few biosynthetically radio-labelled components were immunogenic as revealed by the results of immune-coprecipitation in this study. In the event that some ES components might not be utilising methionine as their precursor, future biosynthetic labelling studies could use other amino acids such as leucine, isoleucine or cysteine. Unlabelled ES of flukes could be radioiodinated and used in immunecoprecipitation. The results of this study could then be compared with the results obtained from immunecoprecipitation of biosynthetically radio-labelled ES. Any antigens detected in the radioiodinated ES but not in the biosynthetically radio-labelled ES might either be utilising other amino acids as their precursors or might be surface components released into the ES.

It would be useful to probe unlabelled and biosynthetically radio-labelled ES of various ages of flukes with sera from infected resistant (cattle) and infected susceptible (sheep and rabbits) hosts, and compare similarities and differences. This may shed further light as to components that are likely to be important in protection.

A major problem encountered in this study was the non-specific precipitation of a major component (about 30 kDa) by normal rat serum. This means that the goat anti-rat gammaglobulin is reacting with components in the normal rat serum. Further immuno-coprecipitation studies should be tried using protein G as the precipitating second antibody.

Most of the immunogenic components identified in this trial (i.e 25 kDa doublet in western blots of D14 ES; 25, 26 and 27 kDa in western blots of adult ES; 26 and 38 kDa respectively in biosynthetically radio-labelled D1 and parenchymal ES (D14, D28 and D42) had low molecular weight. It is therefore suggested that future studies should be done on lower gel gradients (e.g 5-10%). For instance, the

38 kDa recognised in biosynthetically radio-labelled ES of parenchymal flukes as well as the 26 and 27 kDa antigens recognised in western blots of non-reduced adult ES may possibly contain more than one component and separation on a low gradient gel might reveal more information on the make-up of the antigens. In addition, separation of unlabelled ES for western blots as well as immuneprecipitates on 2D eletrophoresis will shed further light on composition of the antigens identified in this study.

Monoclonal antibodies produced against non-immunogenic components detected in biosynthetically radio-labelled ES of flukes may be useful in diagnostic assays designed to detect parasite products. These components are detectable in Table 4.10 but the prominent ones among them includes components of 103, 155 and 168 kDa in D1 ES; 46 kDa in ES of D14 and D28 flukes and 119 kDa in ES of D42 flukes. The antigens (25, 38, 48 and 62 kDa) detected in surface detergent (NoG) strips of adult fluke could be of diagnostic value. As discussed already, the recognition of these antigens by sera collected earlier in infection might indicate that such components may also be present on the surfaces of younger flukes. Monoclonal antibodies against them could therefore be useful in early diagnosis of infection.

The antigens revealed in passive protection trial 2 as apparently being involved in protection merit further investigation. These include the 191 kDa antigen in D0 and D1 ES, 25 kDa doublet antigen in D14 ES, and 25 kDa, 27 and 26 kDa in reduced and non-reduced adult ES respectively. These antigens could be isolated, purified, possibly cloned and used in laboratory immunization trials. Similarly, antigens recognised in biosynthetically radio-labelled ES of D1 (26 kDa) and parenchymal (38 kDa) flukes could be purified and their cDNA cloned, and tested in immunization trials.

REFERENCES

Agadir, H., Haroun, E.M. and Gameel, A.A. (1987). The protective effect of irradiated metacercariae of *Fasciola gigantica* against homologous challenge in sheep. *Journal of Helminthology*, **61**: 137-142.

Ajanusi, O.J., Ogunsusi, R.A., Njoku, C.O. and Gyang, E.O. (1988). *Fasciola gigantica*: Pathological and helminthological observations in experimental infection of *Yankassa lambs. Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **41**: 381-386.

Ajanusi, O.J. (1993). Unpublished results.

Anderson, J.C., Hughes, D.L. and Harness, E (1975). The immune response of rats to subcutaneous implantation with *Fasciola hepatica. British Veterinary Journal*, **131**: 509-516.

Armour, J. and Dargie, J.D. (1973). Studies on immunity to *Fasciola hepatica. Annali della Facolta di Medicina Veterinaria di Torino*, 30 (supplement): 135-137.

Armour, J. and Dargie, J.D. (1974). Immunity to *Fasciola hepatica* in the rat: successful transfer of immunity by lymphoid cells and serum *Experimental parasitology*, **35**: 381-388.

Armour, J. and Dargie, J.D., Doyle, J.J., Murray, M., Robinson, P. and Rushton, B. (1974). Immunisation against fascioliasis. *Proceedings of the Third International Congress of Parasitology*, Munich; 494.

Auriault, C., Ouiassi, M.A., Torpier, G., Eisen, H. and Capron, A. (1980). Proteolytic cleavage of IgG bound to the receptor of *Schistosoma mansoni* schistosomula. *Parasite Immunology*, **3**: 33-44.

Baalawy, S.S. (1975). Studies on immune mechanism of *Fasciola gigantica* infection in rabbits. *Bulletin of Animal Health and Production in Africa*, **23**: 99-102.

Babalola, D.A. and Schillhorn Van Veen, T.W. (1976). Incidence of fascioliasis in cattle slaughtered in Bauchi (Nigeria). *Tropical Animal Health and Production*, **8**: 243-247.

Badley, J.E., Grieve, R.B., Rockey, J.H. and Glickman, L. T. (1987). Immune-mediated adherence of eosinophils to *Toxocara canis* infective larvae: the role of excretory-secretory antigens. *Parasite Immunology*, **9**: 133-143.

Balloul, J.M., Grzych, J.M., Pierce, R.J., and Capron, A. (1987). A purified 28,000 daltons protein from *S. mansoni* worms protects rats and mice against experimental schistosomiasis mansoni. *Journal of Immunology*, **138**: 3448.

Bancroft, J.D. and Stevens, A. (1977). *Theory and practice of Histological Techniques*. Churchill Livingstone, Edinburgh.

Befus, A.D. and Bienenstock, J. (1982). Factors involved in symbiosis and host resistance at the mucous-parasite surface. *Progress in Allergy*, **31**: 76-177.

Bennett, C.E. and Threadgold, L.T. (1973). Electron microscope studies of *Fasciola hepatica*. XIII. Fine structure of newly excysted juvenile. *Experimental Parasitology*, **34**: 85-99.

Bennett, C.E. and Threadgold, L.T. (1975). *Fasciola hepatica*: Development of the tegument during migration in the mouse. *Experimental Parasitology*, **38**: 38-55.

Bennett, C.E. (1978). The identification of soluble adult antigen on the tegumental surface of juvenile *Fasciola hepatica*. *Parasitology*, **77**: 325-332.

Bennett, C.E., Hughes, D.L. and Harness, E. (1980). *Fasciola hepatica*: Changes in tegument during killing of adult flukes surgically transferred to sensitized rats. *Parasite Immunology,* **2**: 39-55.

Bennett, C.E., Joshua, G.W. and Hughes, D.L. (1982). Demonstration of juvenile-specific antigens of *Fasciola hepatica*. *Journal of Parasitology*, **68**: 791-795.

Bitakaramire, P.K. (1973). Preliminary studies on the immunization of cattle against fascioliasis using gamma-irradiated metacercariae of *Fasciola gigantica*. In '*Isotope and Radiation in Parasitology*' **iii**, 23-32.

Bolbol, A.S. (1975). The immunology of fascioliasis in rabbits. Ph.D. Thesis, University of Edinburgh.

Bolbol, A.S., Hammond, J.A. and Sewell, M.M.H. (1978). The response of rabbits to repeated infections with *Fasciola hepatica*. *Veterinary Science Communications*, **2**: 231-235.

Boray J.C. (1967). Studies on experimental infections with *Fasciola hepatica*, with particular reference to acute fascioliasis in sheep. *Annals of Tropical Medicine and Parasitology*, **61**: 439-450.

Boray J.C. (1969). Experimental fascioliasis in Australia. *Advances in Parasitology*, **7**: 95-210.

Bremner, K.C., Ogilvie, B.M. and Keith, R.K. and Berrie, D.A. (1973). Acetylcholine esterase secretion by parasitic neamtodes. III. *Oesophagostomum* spp. *International Journal for Parasitology*, **3**: 609-618.

Burden, D.J. and Hammet, N.C. (1980). *Fasciola hepatica*: Attempts to immunise rats using fluke eggs and *in vitro* culture products. *Veterinary Parasitology*, **7**: 51-57.

Burden, D.J., Harness, E. and Hammet, N.C. (1982). *Fasciola hepatica*: Attempts to immunise rats and mice with metabollic and somatic antigens derived from juvenile flukes. *Veterinary Parasitology*, **9**: 261-266.

Burger, C.J., Rikihisa, Y. and Lin, Y.C. (1986). *Taenia taeniaformis* inhibition of mitogen induced proliferation and interleukin 2 production in rat splenocytes by larval *in-vitro* product. *Experimental Parasitology*, **62**: 216-222.

Butterworth, A.E., Remold, H.G., Houba, V., David, P.H. and Sturrock, R.F. (1977). Antibody-dependent eosinophil-mediated damage to ^{51}Cr-labelled schistosomula of *Schistosoma mansoni*: mediation by IgG, and inhibition by antigen-antibody complexes. *Journal of Immunology*, **118**: 2230-2236.

Butterworth, A.E. (1984). Cell-mediated damage to helminths. *Advances in Parasitology*, **23**:143-235.

Capron, M., Torpier, G. and Capron, A. (1979). *In vitro* killing of *S. mansoni* schistosomula by eosinophils from infected rats: role of cytophilic antibodies. *Journal of Immunology*, **123**: 2220-2230.

Campbell, N.J., Gregg, P., Kelly, J.D. and Dineen, J.K. (1978). Failure to induce homologous immunity in sheep vaccinated with irradiated metacercariae. *Veterinary Parasitology*, **4**: 143-152.

Chapbell, C.L. and Dresden, M.H. (1986). *Schistosoma mansoni*: Proteinase activity of "hemoglobinase" from the digestive tract of adult worms. *Experimental Parasitology*, **61**: 160-167.

Chapman, C.B. and Mitchell, G.F. (1982a). *Fasciola hepatica* : Comparative studies on fasciolosis in rats and mice. *International Journal of parasitology*, **12**: 81-91.

Chapman, C.B. and Mitchell, G.F. (1982b) Proteotylic cleavage of immunoglobulin by enzymes released by *Fasciola hepatica*. *Veterinary Parasitology*, **11**: 165-178.

Chick, B.F. (1979). Economic significance of *Fasciola hepatica* infestation in beef cattle - a definitive study based on field trial and grazier questionaire. In *Proceedings of the Second International Symposium on Veterinary Epidemiology and Economics*, Canberra, Australia, 377-382. Edited by W. A. Goernig, R. T. Roe and L. A. Chapman.

Chick, B.F., Coverdale, D.R. and Jackson, A.R.B. (1980). Production effects of liver fluke (*Fasciola hepatica*) infection in beef cattle. *Australian Veterinary Journal*, **56**: 588-592.

Colligan, E.J., Kruisbeek, M.A., Margulies. D.H., Shevach, M.E. and Strober, W. (1992). Current Protocols in Immunology, 8.12.1. Greene Publishing and Wiley Interscience, New York.

Corba, J., Armour, J., Roberts, R.J. and Uquhart, G.M. (1971). Transfer of immunity to *Fasciola hepatica* infection by lymphoid cells. *Research in Veterinary Science*, **12**: 292-295.

Dalton, P.J. and Joyce, P. (1987). Characterization of surface glycoproteins of different developmental stages of *Fasciola hepatica* by surface radiolabelling. *Journal of Parasitology*, **73**: 1281-1284.

Dalton, P.J., Tom, T.D. and Strand, M. (1985). *Fasciola hepatica*: Comparison of immature and mature immunoreactive glycoproteins. *Parasite Immunology*, **7**: 643-657.

Dargie, J.D., Armour, J. and Uquhart, G.M (1973). Studies on immunity to *Fasciola hepatica*. *Parasitology*, **67**: xxv.

Dargie, J.D., Armour, J., Rushton, B. and Murray, M. (1974). Immune mechanisms and hepatic fibrosis in fascioliasis. *Proceedings of the Sixth International Conference for the Advancement of Veterinary Parasitology*, Vienna, 249-272. Edited by E. J. L. Soulsby in Parasitic Zoonoses. Academic Press Inc., New York and London.

Dargie, J.D., Berry, C.I. and Parkins J.J. (1979). The patho-physiology of ovine fascioliasis: studies on the feed intake and digestibility, body weight and nitrogen balance of sheep given rations of hay or hay plus a pelleted supplement. *Research in Veterinary Science*, **76**: 289-295.

Davies, C., Rickard, M.D., Smyth, J.D. and Hughes, D.L. (1979). Attempts to immunize rats against infection with *Fasciola hepatica* using *in vitro* culture antigens from newly excysted metacercariae. *Research in Veterinary Science*, **26**: 259-260.

Davies, C. and Goose, J. (1981). Killing of newly excysted juveniles of *Fasciola hepatica* in sensitised rats. *Parasite Immunology*, **3**: 81-86.

Dawes, B. (1962). Additional notes on growth of *Fasciola hepatica* L. in the mouse, with some remarks about recent researches in Belgium. *Journal of Helminthology*, **36**: 259-268.

Dawes, B. (1964). A preliminary study of the prospect of inducing immunity in fascioliasis by means of infections with X-irradiated metacercarial cysts and subsequent challenge with normal cysts of *Fasciola hepatica* L. *Parasitology*, **54**: 369-389.

Dawes, B. and Hughes, D.l. (1964). Fascioliasis: The invasive stages of *Fasciola hepatica* in mammalian hosts. *Advances in Parasitology*, **2**: 97-168.

Dawes, B. and Hughes, D.l. (1970). Fascioliasis. The invasive stages in mammals. *Advances in Parasitology*, **8**: 259-270.

De Weil, N.S, Hillyer, G.V and Pacheco, E. (1984). Isolation of *Fasciola hepatica* genus specific antigens *International Journal of Parasitology*, **14**(2): 197-206.

Despommier, D.D. and Muller, M. (1976). The stichosome and its secretion granules in the mature muscle larva of *Trichinella spiralis*. *Journal of Parasitology*, **62**: 775-785.

Diaw, D.T., Seya, M. and Sarr, Y. (1988). Epidemiology of trematodiasis in livestock in the Kolda region, Casamance (senegal). *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **41**: 257-264.

Doy, T.G., Hughes, D.L and Harness, E (1978). Resistance of the rat to infection with *Fasciola hepatica* and the possible involvement of intestinal eosinophil leucocytes. *Research in Veterinary Science*, **25**: 41-44.

Doy, T.G. and Hughes, D.L. (1982). Evidence of two distinct mechanisms of resistance in the rat to reinfection with *Fasciola hepatica*. *International Journal for Parasitology*, **12**: 357-361.

Doy, T.G. and Hughes, D.L. (1984). *Fasciola hepatica:* Site of resistance to reinfection in cattle. *Experimental Parasitology*, **57**: 274-278.

Doyle, J.J. (1971). Acquired immunity to experimental infection with *Fasciola hepatica* in cattle. *Research in Veterinary Science*, **30**: 294-297.

Doyle, J.J. (1973). The relationship between the duration of a primary infection and the subsequent development of an acquired resistance to experimental infections with *Fasciola hepatica* in calves. *Research in Veterinary Science*, **14**: 97-103.

Eckblad, P.W., Woodard, F.L and Lang, B.Z. (1981). Scanning electron microscopy of *in vitro* serum mediated destruction of juvenile *Fasciola hepatica*. *Journal of Parasitology*, **67**: 784-789.

Edwards, A.J., Burt, J.S. and Ogilvie, B.M. (1971). The effect of immunity upon some enzymes of the parasitic nematode *Nippostrongylus brasiliensis*. *Parasitology*, **62**: 339-347.

Eisen, H. and Tallan, I. (1977). *Tetrahymena pyriformis* recovers antibody immobilization by producing univalent antibody fragments. *Nature*, **270**: 514-515.

Ershov, V.S. (1959). The problem of immunisation of domestic animals to helminthoses. *Proceedings of the 16th International Veterinary Congress*, Madrid, 279-291.

Ericksen, L. and Flagstad, T. (1974). *Fasciola hepatica*: Influence of extrahepatic adult flukes on infection and immunity in rats. *Experimental Parasitology*, **35**: 411-417.

Fabiyi, J.P., Adeleye, G.A. (1982). Bovine fascioliasis on the Jos Plateau, Northern Nigeria, with particular reference to economic importance. *Bulletin of Epizootic Diseases of Africa*, **30**: 41-43.

Fabiyi, J.P. (1986). Production losses and control of helminths in ruminants of tropical regions. *International Journal of Parasitology*, **17**: 435-442.

Forsyth, K.P., Spark, R., Kazura, J., Brown, G.V., Peters, P., Heywood, P., Dissanayake, S. and Mitchell, G. F. (1985). A monoclonal antibody-based immunoradiometric assay for detection of circulating antigen in bancroftian filariasis. *Journal of Immunology*, **134**: 1172-1177.

Fortmeyer, H.P. (1973). Immunological studies on *Fasciola hepatica* infections of rabbits. *Deutche Tierarztliche Wochenschrift*, **80**: 528-534.

Fortmeyer, H.P. (1974). Extrahepatic immunity in *Fasciola hepatica* infections in rabbits. *Deutche Tierarztliche Wochenschrift*, **81**: 356-359.

Fowler, J. and Cohen, L. (1990). *Practical statistics for Field Biology*. Open University Press, Milton Keynes.

Froyd, G. (1978). Fascioliasis in cattle and sheep. In: *Fourth international Congress of Parasitology*, Warsaw, Poland, 81-82.

Gamble, H.R. (1985). *Trichinella spiralis*: Immunization of mice using monoclonal antibody affinity-isolated antigens. *Experimental Parasitology*, **59**: 398-404.

Goose, J. and MacGregor, M. (1973). Naturally acquired immunity to *Fasciola hepatica* in the rat. *British Veterinary Journal*, **129**: XLIX.

Goose, J. and MacGregor, M. (1974). Influence of age and sex on rat susceptibility to *Fasciola hepatica* infection. *Parasitology*, **69**: xvii

Goose, J. (1978). Possible role of excretory/secretory products in evasion of host defences by *Fasciola hepatica*. *Nature*, **275**: 216-217.

Gorgi, J.R. and Theodorides, V.J. (1980). Parasitology for Veterinarians. 3rd ed., 58-60. W.B. Saunders Company, Philadelphia, London and Toronto.

Gottstein, B. (1985). Purification and characterization of a specific antigen from *Echinococcus multilocularis*. *Parasite Immunology*, 7: 201-212.

Gretillat, S. (1961). Note preliminaire sur l'epidemiologie de la distomatose bovine en Senegal. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **14**: 283-291.

Guralp, N., Ozcan, C. and Simms, B.T. (1964). *Fasciola gigantica* and fascioliais in Turkey. *American Journal of Veterinary Research*, **25**: 196-210.

Gundlach, J.L. (1971). A study of the phenomena of immunity in the course of experimental fascioliasis in rabbits. *Acta Parasitologica Polonica*, **19**: 285-306.

Hall, R.F. and Lang, B.Z. (1978). The development of an experimental vaccine against *Fasciola hepatica* in cattle. Proceedings of 82nd Annual Meeting of US Animal Health Association, Buffalo, New York.

Hames, B.D. and Rickwood, D. (1990). In: *Gel Electrophoresis of Proteins. A pratical approach*. Second edition. Edited by B. D. Hames and Rickwood. Pg 85.

Hammond, J.A. (1972). Infections with *Fasciola* spp. in wild life in Africa. *Tropical Animal Health and Production*, **4**: 1-13.

Hang, L.M., Warren, K.S. and Boros, D.L. (1974). *Schistosoma mansoni*: antigenic secretions and the etiology of egg granulomas in mice. *Experimental Parasitology*, **35**: 288-298.

Hanna, R.E.B. (1976). *Fasciola hepatica*: A light and electron microscope autoradiographic study of shell-protein and glycogen synthesis, by vitteline follicles in tissue slices. *Experimental Parasitology*, **39**: 18-28.

Hanna, R.E.B. (1979). Surface antigenicity of *Fasciola hepatica* during development. *Parasitology*, **79**: xxxi.

Hanna, R.E.B. (1980a). *Fasciola hepatica*: Glycocalyx replacement as a possible mechanism for protection against host immunity. *Experimental Parasitology*, **50**: 103-114.

Hanna, R.E.B. (1980b). *Fasciola hepatica*: Autoradiography of protein synthesis, transport and secretion by the tegument. *Experimental Parasitology*, **50**: 297-304.

Harness, E., Hughes, D.L and Doy, T.G. (1976). The demonstration of pre-hepatic immune response to *Fasciola hepatica* in the mouse. *International Journal of Parasitology*, **6**: 15-17.

Harness, E., Doy, T.G. and Hughes, D.L (1977). The early migratory behaviour of young *Fasciola hepatica* in sensitized mice. *International Journal of Parasitology*, **7**: 51-54.

Haroun, E. (1979). Studies on resistance to *Fasciola hepatica* in rats and rabbits. Ph.D. Thesis, University of Edinburgh.

Haroun, E., Hammond, J.A and Sewell, M.M.H. (1980a). Resistance of *Fasciola hepatica* in rats and rabbits following implantation of adult flukes contained in diffusion chambers. *Research in Veterinary Science*, **29**: 310-314.

Haroun, E., Hammond, J.A and Sewell, M.M.H. (1980b). Resistance to *Fasciola hepatica* in rats and rabbits following sensitising infection and treatment. *Research in Veterinary Science*, **28**: 377-379.

Haroun, E.M., Hammond, J.A and Sewell, M.H.H. (1981). Passive transfer of humoral resistance to *Fasciola hepatica* in the rats and rabbits. *Research in Veterinary Science*, **30**: 309-311.

Harnett, W., Meghji, M., Worms, M.J. and Parkhouse, R.M.E (1986). Quantitative and qualitative changes in production of excretions/secretions by *Litomosoides carinii* during development in the jird (*Meriones unguiculatus*). *parasitology*, **93**: 317-331.

Harrison, L.J.S., Parkhouse, R.M.E. and Sewell, M.M.H. (1984). Variation in 'target' antigens between appropriate and inappropriate hosts of *Taenia saginata* metacestodes. *Parasitology*, **88**: 659-663.

Harrison, L.J.S. and Parkhouse, R.M.E. (1986). Passive protection against *Taenia saginata* in cattle by a mouse monoclonal antibody reactive with the surface of the invasive onchosphere. *Parasite Immunology*, **8**: 319-332.

Hawkins, C.D. and Morris, R.S. (1978). Depression of productivity in sheep infected with *Fasciola hepatica*. *Veterinary Parasitology*, **4**: 341-352.

Hayes, T.J., Bailer, J. and Mitrovic, M. (1972). Immunity in rats to superinfection with *Fasciola hepatica*. *The Journal of Parasitology*, **58**: 1103-1105.

Hayes, T.J., Bailer, J. and Mitrovic, M. (1973). Immunity to *Fasciola hepatica* in rats: The effect of two different levels of primary exposure on superinfection. *Journal of Parasitology*, **59**: 810-812.

Hayes, T.J., Bailer, J. and Mitrovic, M. (1974a). Serum transfer of immunity to *F. hepatica* in rats. *Journal of Parasitology*, **60**: 722-723.

Hayes, T.J. Bailer, J. and Mitrovic, M. (1974b). Studies on serum transfer of immunity to *Fasciola hepatica* in the rat. *The Journal of Parasitology*, **60**: 930-934.

Hayes, T.J., Bailer, J. and Mitrovic, M. (1974c). Acquired immunity and age resistance in rats with chronic fascioliasis. *The Journal of Parasitology*, **60**: 247-250.

Hayes, T.J. and Mitrovic, M. (1977). The early expression of protective immunity to *Fasciola hepatica* in rats. *Journal of Parasitology*, **63**: 584-587.

Healy, G.R. (1955). Studies on immunity to *Fasciola hepatica* in rabbits. *Journal of Parasitology*, **41** (Supplement): 25.

Heath, D.D., Lawrence, S.B., Glennie, A. and Twaalhoven, H. (1985). The use of excretory and secretory antigens of the scolex of *Taenia ovis* for the serodiagnosis of infection in dogs. *Journal of Parasitology*, **71**: 192-199.

Henderson, W.W. (1936). Diseases of sheep and goats: liver fluke (Fascioliasis). *Annual Report, Veterinary Department of Nigeria*, 15.

Hillyer, G. V. (1980). Isolation of *Fasciola hepatica* tegument antigens. *Journal of Clinical Microbiology*, **12**: 695-699.

Hillyer, G.V. (1985). Induction of immunity in mice to *Fasciola hepatica* with a *Fasciola/Schistosoma* cross-reactive defined immunity antigen. *American Journal of Tropical Medicine and Hygiene*, **34**: 1127-1131.

Hillyer, G.V. and Serrano, A.E. (1986). Fractionation of *Fasciola hepatica* tegument antigens and their application to the serodiagnosis of experimental fascioliasis by the enzyme-linked immunosorbent assay. *Journal of Helminthology*, **60**: 173-178.

Hillyer, G.V., Haroun, E.M., Hernandez, A. and Soler De Galanes, M. (1987). Acquired resistance to *Fasciola hepatica* in cattle using purified adult worm antigen. *American Journal of Tropical Medicine and Hygiene*, **37**: 363-369.

Hillyer, G.V. and Taylor, D.W. (1988). Immunoprecipitation of *Fasciola hepatica* mRNA *in vitro* translation products using infection and hyperimmune sera. *American Journal of Tropical Medicine and Hygiene*, **38**: 547-552.

Hillyer, G.V. and Soler De Galanes, M. (1988). Identification of a 17-Kilodalton *Fasciola hepatica* immunodiagnostic antigen by the Enzyme-Linked Immunoelectrotransfer Blot Technique. *Journal of Clinical Microbiology*, **26**: 2048-2053.

Homan, W.L., Derksen, A.C.G., Knapen, F.V. and Van-Knapen, F. (1992). Identification of diagnostic antigens from *Trichinella spiralis*. *Parasitology Research*, **78**(2): 112-119.

Hotez, P.J. and Cerami, A. (1983). Secretion of proteolytic anticoagulant by *Ancylostoma* hookworms. *Journal of Experimental Medicine*, **157**: 1594-1603.

Hotez, P.J., LE Trang, N., McKerrow, J.H and Cerami, A. (1985). Isolation and characterization of a proteolytic enzyme from the adult hookworm *Ancylostoma caninum*. *Journal of Biological Chemistry*, **26**: 7343-7348.

Howell, M.J. (1979). Vaccination of rats against *Fasciola hepatica*. *Journal of Parasitology*, **65**: 817-819.

Howell, M.J., Sandeman, R.M. and Rajasekariah, G.R. (1977). *In vivo* and *in vitro* studies on the effects of immune rat serum on *Fasciola hepatica*. *International Journal of Parasitology*, **7**: 367-371.

Howell, M.J. and Sandeman, R.M. (1979). *Fasciola hepatica*: Some properties of a precipitate which forms when metacercariae are cultured in immune rat serum. *International Journal of Parasitology*, **9**: 41-45.

Hughes, D.L. (1962). Observations on the immunology of *Fasciola hepatica* infections in mice and rabbits. *Parasitology*, 52: 4

Hughes, D.L., Anderson, J.C. and Harness, E. (1976). *Fasciola hepatica*: The effect of challenge by the subcutaneous or intraperitoneal route with living adult flukes in sensitized rats. *Experimental Parasitology*, **40**: 355-362.

Hughes, D.L., Hanna, R.E.B., Symonds, H.W. (1981). *Fasciola hepatica*: IgG and IgA levels in the serum and bile of infected cattle. *Experimental Parasitology*, **52**: 271-279.

Hughes, D.L., Harness, E. and Doy, T.G. (1981). The different stages of *Fasciola hepatica* capable of inducing immunity and the susceptibility of various stages to immunological attack in the sensitized rat. *Research in Veterinary Science*, **30**: 93-98.

Hughes, D.L., Doy, T.G., Burden, D.J. and Oldham, G. (1982). Stage-specific immunity in the rat as demonstrated by τ-irradiated *Fasciola hepatica*. *Parasitology*, **84**: xiii.

Hunter A.G. and Health, P.J. (1984). Ovine internal parasitism in the Yemen Arab Republic. *Tropical Animal Health and Production*, **16**: 95-106.

Irving, D.O. and Howell, M.J. (1982). Characterization of excretory-secretory antigens of *Fasciola hepatica*. *Parasitology*, **85**: 179-188.

Janssen, D., Wit, M., Rycke, P.H., De-Wit, M., De-Rycke, P.H. (1990). Hydatidosis in Belgium: analysis of larval Echinococcus granulosus by SDS-PAGE and western blotting. *Annales de la Societe Belge de Medecine Tropicale*, **70**(2): 121-129.

Jenkins, D.J. and Rickard, M.D. (1986a). Specificity of scolex and and onchosphere antigens for the serological diagnosis of taeniid cestode infections in dogs. *Australian Veterinary Journal*, **63**: 40-42.

Jenkins, D.J. and Rickard, M.D. (1986b). Specific antibody responses in dogs experimentally infected with *Echinococcus granulosus*. *American Journal of Tropical Medicine and Hygiene*, **35**: 345-349.

Jenkins, S.N. and Wakelin, D. (1977). The source and nature of some functional antigens of *Trichuris muris*. *Parasitology*, **74**: 153-161.

Jones, V.E., Edwards, A.J. and Ogilvie, B.M. (1970). The circulating immunoglobulins involved in protective immunity to the intestinal stage of *Nippostrongylus brasilensis*. *Immunology*, **18**: 621-633.

Joshua, G.W.P., Harrison, L.J.S. and Sewell, M.M.H. (1988). Excreted/secreted products of developing *Taenia saginata* metacestodes. *Parasitology*, **97**: 477-487.

Karib, E.A. (1962). Fascioliasis in cattle and sheep in the Sudan. *Bulletin Office International des Epizooties*, **58**: 337-346.

Kazura, J.W. and Meshnick, S.R. (1984). Scavenger enzymes and resistance to oxygen mediated damage in *Trichinella spiralis*. *Molecular and Biochemical Parasitology*, **10**: 1-10.

Keegan, P.S. and Trudgett, A. (1992). *Fasciola hepatica* in the rat: Immune responses associated with the development of resistance to infection. *Parasite Immunology*, **14**: 657-669.

Kendall, S.B. (1954). Fascioliasis in Pakistan. *Annals of Tropical Medicine and Parasitology*, **48**: 307-313.

Kendall, S.B. (1967). Host specificity as evidenced by species of *Fasciola*. *Helminthologia*, **8**: 223-233.

Kendall, S.B., Hebert, N., Partiff and Pierce, M.A. (1967). Resistance to reinfection with *Fasciola hepatica* in rabbits. *Experimental Parasitology*, **20**: 242-247.

Kendall, S.B.and Sinclair, I.J. (1971). Barriers to reinfection with *Fasciola hepatica* in the rabbit. *Research in Veterinary Science*, **12**: 74-79.

Kendall, S.B., Sinclair, I.J., Everett, G. and Partiff, J.W (1978). Resistance to *Fasciola hepatica* in cattle. 1. Parasitological and serological observations. *Journal of Comparative Pathology*, **88**: 115-122.

Kerr, K.B. and Petkovich, O.L. (1935). Active immunity in rabbits to the liver fluke, *Fasciola hepatica*. *Journal of Parasitology*, **21**: 319-320.

Kusel, J.R., MacKenzie, P.E. and McLaren D.J. (1975). The release of membrane antigens into culture by adult *Schistosoma mansoni*. *Parasitology*, **71**: 247-259.

Kwan-Lim, G.E., Gregory, W.F., Selkirk, M.E., Partono, F. and Maizels, R.M. (1989). Secreted antigens of filarial nematodes: a survey and characterization of *in vitro* excreted/secreted products of adult *Brugia malayi*. *Parasite Immunology*, **11**: 629-654.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, **227**: 680-685.

Lammas, D.A. and Duffus, W.P.H. (1985). Identification of surface proteins of juvenile stages of *Fasciola hepatica*. *Research in Veterinary Science*, **38**: 248-249.

Lammler, G. (1959). Die chemotherapie der Fasciolose III. Mitteilung. Uber die experimentelle *Fasciola hepatica* infektion der Albino-Ratte. *Zeitschrift fur Tropenmedicin und Parasitologie*, 10: 379-384.

Lang, B.Z. (1967). Host-parasite relationships of *Fasciola hepatica* in the white mouse. II. Studies on acquired immunity. *The Journal of Parasitology*, **53** (1): 21-30.

Lang, B.Z., Larsh, J.E., Weatherly, F.N. and Goulson, T.H. (1967). Demonstration of immunity to *Fasciola hepatica* in recipient mice given peritoneal exudate cells. *The Journal of Parasitology*, **53**: 208-209.

Lang, B.Z. (1968). Acquired immunity to *Fasciola hepatica* in the white mouse. *American Journal of Tropical Medicine and Hygiene*, **17**: 561-567.

Lang, B.Z. and Dronen, N.O. (1972). Host-parasite relationships of *Fasciola hepatica* in the white mouse IV. Studies on worm transfer and acquired immunity by worms of different ages. *The Journal of Parasitology*, **58**: 84-87.

Lang, B.Z. (1974a). Host parasite relationship of *Fasciola hepatica* in the white mouse. V. Age of fluke responsible for the induction of acquired immunity. *The journal of Parasitology*, **60**: 90-92.

Lang, B.Z. (1974b). Host parasite relationship of *Fasciola hepatica* in the white mouse. VI. Studies on the effects of immune and normal sera on the viability of young worms transferred to normal recipient. *The Journal of Parasitology*, **60**: 925-929.

Lang, B.Z. (1976). Host parasite relationship of *Fasciola hepatica* in the white mouse. VII. Effects of antiworm incubate sera on transferred worms and successful vaccination with a crude antigen. *Journal of Parasitology*, **62**: 232-236.

Lang, B.Z. and Hall, F.R. (1977). Host parasite relationships of *Fasciola hepatica* in the white mouse. VII. Successful vaccination with culture sonic disruption of immature worms. *Journal of Parasitology*, **63**: 1046-1049.

Lehner, R.P (1977). *In vitro* culture of *Fasciola hepatica* and the immunology associated with the metabolic products of the trematode. Ph.D Thesis, University of Edinburgh.

Lehner, R.P. and Sewell, M.M.H. (1979). Attempted immunization of laboratory animals with metabolic antigens of *Fasciola hepatica*. *Veterinary Science Communication*, **2**: 337-340.

Lehner, R.P and Sewell M.M.H (1980). A study of the antigens produced by adult *F. hepatica* maintained *in vitro*. *Parasite Immunology*, **2**: 99-109.

Lemma, B., Gabre-Ab, F. and Telda, S. (1985). Studies on Fascioliasis in four selected sites in Ethiopia. *Veterinary Parasitology*, **18**: 29-37.

Letonja, T. and Hammerberg, C. (1987). *Taenia taeniaformis*: early inflammatory response around developing metacestodes in the liver of resistant and susceptible mice. II. Histochemistry and cytochemistry. *Journal of Parasitology*, **73**: 971-

Lightlowers, M.W. and Rickard, M.D. (1988). Excretory-secretory products of helminth parasites: effects on host immune responses. *Parasitology*, **96**: S123-S166.

Lindquist, R.N., Senft, A.W., Petit, M and McKerrow, J.H. (1986). *Schistosoma mansoni*: Purification and characterization of major acidic proteinase from adult worms. *Experimental Parasitology*, **61**: 398-404.

Maizels, R.M., Denham, D.A. and Sutanto, I. (1985). Secreted and circulating antigens of the filarial parasite *Brugia pahangi*: analysis of *in vitro* released components and detection of parasite products *in vivo*. *Molecular and Biochemical Parasitology*, **17**: 277-288.

Mazingue, C., Camus, D., Dessaint, J.P., Capron, M. and Capron, A. (1980). *In vitro* and *in vivo* inhibition of mast cell degranulation by a factor from *Schistosoma mansoni*. *International Archives of Allergy and Applied Immunology*, **63**: 178-189.

Mckerrow, J.H., Pino-Heiss, S., Lindquist, R. and Werb, Z. (1985). Purification and characterization of an elastinolytic proteinase secreted by cercariae of *Schistosoma mansoni*. *Journal of Biological Chemistry*, **260**: 3703-3707.

Milbourne E.A and Howell M.J. (1990). Eosinophil responses to *Fasciola hepatica* in rodents. *International Journal of Parasitology*, **20**: 705-708.

Mitchell, G.B.B., Armour, J., Ross, J.G., Halliday, W.G. (1981). Successful transfer of resistance to *Fasciola hepatica* infections in rats by immune serum and transfer factor. *Research in Veterinary Science*, **30**: 246-247.

Moqbel, R. and MacDonald, A.J. (1990). Immunological and inflammatory responses in the small intestine associated with helminth infections. In: *Parasites: Immunity and Pathology. The consequences of parasitic infection in mammals*. 249-282. Edited by Behnke, J.M. Taylor and Francis Ltd., London.

Moloney, A. and Denham, D.A. (1979). Effects of immune serum and cells on newborn larvae of *Trichinella spiralis*. *Parasite Immunlogy*, **1**: 3-12.

Morrisey, J.H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry*, **117**: 307-310.

Mzembe, S.A.T. and Chaudhry, M.A. (1980). The epidemiology of fascioliasis in Malawi. Part II. Epidemiology in the definitive host. *Tropical Animal Health and Production*, **13**: 27-33.

Moore, M.N. and Halton, D.W. (1976). *Fasciola hepatica*: Histochemical observations on juvenile and adults and the cytopathological changes induced in infected mouse liver. *Experimental Parasitology*, **40**: 212-224.

Nansen, P. (1975). Resistance in cattle to *Fasciola hepatica* induced by τ-ray attenuated larvae: results from a controlled field trial. *Research in Veterinary Science*, **19**: 278-283.

Ogambo-Ongoma, A.H. (1972). Fascioliasis survey in Uganda. *Bulletin of Epizootic Diseases in Africa*, **20**: 35-41.

Oldham, G. and Hughes, D.L. (1982). *Fasciola hepatica*: Immunization of rats by intraperitoneal injection of adult fluke antigen in Freund's adjuvant. *Experimental Parasitology*, **54**: 7-11.

Oldham, G. (1983). Protection against *Fasciola hepatica* in rats with adult fluke antigen in Freund's complete adjuvant: influence of antigen batch, antigen dose and number of sensitizing injections. *Research in Veterinary Science*, **34**: 240-244.

Oldham, G. (1985). Immune responses in rats and cattle to primary infections with *Fasciola hepatica. Research in Veterinary Science, 39*: 357-363.

Ogilvie, B.M., Rothwell, T.L.W., Bremner, K.C., Schnitzerling, H.J., Nolan, J. and Keith, R.K. (1973). Acetylcholinesterase secretion in parasitic nematodes. I. Evidence for secretion of the enzyme by a number of species. *International journal for Parasitology*, **3**: 589-597.

Ogunrinade, A.F. (1983). Bovine fascioliasis in Nigeria V. The pathogenecity of experimental infections in white Fulani cattle. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **36**: 141-149.

Ogunrinade, A.F. (1984). Infectivity and pathogenicity of *Fasciola gigantica* in West African dwarf sheep and goats. *Tropical Animal Health and Production*, **16**: 161-166.

Ortega-Pierres, G., Mackenzie, C.D. and Parkhouse, R.M.E. (1984). Protection against *Trichinella spiralis* induced by a monoclonal antibody that promotes killing of newborn larvae by granulocytes. *Parasite Immunology*, **6**: 275-284.

Parkhouse, R.M.E. and Clark, N.W.T. (1983). Stage specific secreted and somatic antigens of *Trichinella spiralis*. *Molecular and Biochemical Parasitology*, **9**: 319-327.

Parkhouse, R.M.E. and Ortega-Pierres, G. (1984). Stage specific antigens of *Trichinella spiralis*. *Parasitology*, **88**: 623-630.

Parkhouse, R.M.E., Clark, N.W.T., Maizels, R.M. and Denham, D.A. (1985). *Brugia pahangi*: labelling of secreted antigens with ^{35}S-methionine *in vitro*. *Parasite Immunology*, **7**: 665-668.

Parkhouse, R.M.E., Almond, N.M., Cabrera, Z. and Harnett, W. (1987). Nematode antigens in protection, diagnosis and pathology. *Veterinary Immunology and Immunopathology*, **17**: 313-324.

Parkhouse, R.M.E. and Harrison, L.J.S. (1989). Antigens of parasitic helminths in diagnosis, protection and pathology. *Parasitology*, **99**: S5-S19.

Petralanda, I., Yarzabal, L. and Piessens, W.F. (1986). Studies on filarial antigens with collagenase activity. *Molecular and Biochemical Parasitology*, **19**: 51-59.

Pfister, K., Daveau, C.H. and Ambroise-Thomas, P. (1984). Partial purification of somatic and excretory-secretory products of adult *Fasciola hepatica* and their application for the serodiagnosis of experimental and natural fascioliasis using an ELISA. *Research in Veterinary Science*, **37**: 39-43.

Pfister, K., Turner, K and Wedrychowicz, H. (1984/85). Worm recovery, haemagglutinating antibodies and IgE levels after immunization against *Fasciola hepatica*. *Veterinary Parasitology*, **17**: 139-150.

Poitou, I., Baeza, E. and Boulard, C. (1992). Humoral and cellular response in rats during a primary infection with *Fasciola hepatica*. *Veterinary Parasitology*, **45**: 59-71.

Pritchard, D.I., Crawford, C.R., Duce, I.R. and Behnke, J.M. (1985). Antigen stripping from the nematode epicuticle using the cationic detergent cetyltrimethylammonium bromide (CTAB). *Parasite Immunology*, **7**: 575-585.

Rajasekariah, G.R. and Howell, M.J. (1977). The fate of *Fasciola hepatica* metacercariae following challenge infection of immune rats. *Journal of Helminthology*, **51**: 289-294.

Rajasekariah, G.R. and Howell, M.J. (1978). *Fasciola hepatica*: Role of developmental stages in the rat's resistance to challenge. *Experimental Parasitology*, **44**: 233-238.

Rajasekariah, G.R. and Howell, M.J. (1979). *Faciola hepatica* in rats: Transfer of immunity by serum and cells from infected to *F. hepatica* naive animals. *The Journal of Parasitology*, **65(4)**: 481-487.

Rajasekariah, G.R., Mitchell, G.F., Chapman, C.B., and Montague, P.E. (1979). *Fasciola hepatica*: Attempts to induce protection against infection in rats and mice by injection of excretory/secretory products of immature worms. *Parasitology*, **79**: 393-400.

Rahman, M.A., Ali, A.K. and Rahman, A. (1972). Incidence of disease of cattle in Mymensingh. *Bangladesh Veterinary Journal*, **6**: 25-30.

Ramaniuk, K. (1978). Influence of *Fasciola hepatica* invasion on the reproduction of its final host. In: *Fourth International Congress of Parasitology*, Warsaw, Poland, 22-23.

Reddington, J.J., Wes Leid, R. and Wescott, R.B (1984). A review of the antigens of *Fasciola hepatica*. *Veterinary Parasitology*, **14**: 209-229.

Reid, J.F.S., Doyle, J.J., Armour, J. and Jennings, F.W. (1972). *Fasciola hepatica* infection in cattle. *Veterinary Record*, **90**: 486-487.

Reinhard, E.G. (1957). Landmarks of parasitology 1. The discovery of the life cycle of the liver fluke. *Experimental Parasitology*, **6**: 208-232.

Rhoads, M.L. (1983). *Trichinella spiralis*: identification and purification of superoxide dismutase. *Experimental Parasitology*, **56**: 41-54.

Rickard, M.D. and Bell, J.K. (1971a). Successful vaccination of lambs against infection with *Taenia ovis* using antigens produced during *in vitro* cultivation of the larval stages. *Research in Veterinary Science*, **12**: 401-402.

Rickard, M.D. and Bell, J.K. (1971b). Immunity produced against *Taenia ovis* and *T. taeniaformis* infection in lambs and rats following *in vivo* growth of their larvae in filtration membrane diffusion chambers. *The Journal of Parasitology*, **57**: 571-575.

Rickard, M.D. and Bell, J.K. (1971c). Induction of immunity of lambs to a larval cestode by diffusible antigens. *Nature*, **232**: 120.

Rivera-Marrero, A.C., Santiago, N. and Hillyer, G.V. (1988). Evaluation of immunodiagnostic antigens in the excretory-secretory products of *Fasciola hepatica. Journal of Parasitology*, **74**: 646-652.

Robertson, B.D., Bianco, A.T., McKerrow, J.H. and Maizels, R.M. (1989). *Toxocara canis*: Proteolytic enzymes secreted by the infective larvae *in vitro*. *Experimental Parasitology*, **69**: 30-36.

Roseby, F.B. (1970). The effect of fascioliasis on the wool production in merino sheep. *Australian Veterinary Journal*, **46**: 361-365.

Ross, J.G. (1966). Experimental infection in cattle with *Fasciola hepatica*. *Nature* (London), **212**: 1464-1465.

Ross, J.G. (1967). Studies of immunity to *Fasciola hepatica*: Acquired immunity in cattle, sheep and rabbits following natural infection and vaccine procedures. *Journal of Helminthology*, **41**: 393-399.

Rushton, B. (1977). Ovine fascioliasis following reinfection. *Research in Veterinary Science*, **22**: 133-134.

Sandeman, R.M. and Howell, M.J. (1981). Response of sheep to challenge infection with *Fasciola hepatica*. *Research in Veterinary Science*, **39**: 294.

Santiago, N., Hillyer, G.V., Garcia-Rosa, M. and Morales H.M. (1986). Identification of functional *Fasciola hepatica* antigens in experimental infections in rabbits. *American Journal of Tropical Medicine and Hygiene*, **35**: 135-140.

Santiago, N. and Hillyer, G.V. (1986). Isolation of potential serodiagnostic *Fasciola hepatica* antigens by electroelution from polyacrylamide gels. *American Journal of Tropical Medicine and Hygiene*, **35**: 1210-1217.

Santiago, N. and Hillyer, G.V. (1988). Antibody profiles by EITB and ELISA of cattle and sheep infected with *Fasciola hepatica*. *Journal of Parasitology*, **74**: 810-818.

Schillhorn Van Veen, T.W. (1979). Ovine fascioliasis (*F. gigantica*) on the Ahmadu Bello University Farm. *Tropical Animal Health and Production*, **11**: 151-156.

Schumacher, W. (1938). Untersuchungen uber den Wanderungsweg und die Entwicklung von *Fasciola hepatica* im Endwirt. *Zeitchrift fur Parasitenkunde*, **10**: 608-643.

Sewell, M.M.H. (1964). The immunology of fascioliasis. II. Qualitative studies on the precipitin reaction. *Immunology*, **7**: 671-680.

Sewell, M.M.H. (1966). The pathogenesis of fasciolosis. *The Veterinary Record*, **78** (3): 98-105.

Sewell, M.M.H. (1973). Laboratory notes. Helminthology section, C.T.V.M.

Sewell, M.M.H. (1976). The role of management in the control of helminth diseases. In: *Beef Cattle Production in Developing countries*. 138-149. Edited by A. J. Smith. Lewis Reprints Ltd. Tonbridge.

Sexton, L.J., Milner, A.R. and Campbell, J. (1991). *Fasciola hepatica*: immunoprecipitation analysis of biosynthetically labelled antigens using sera from infected sheep. *Parasite Immunology*, **13**: 105-108.

Sharma, R.L., Dhar, D.N. and Raina O.K. (1989). Studies on the prevalence and laboratory transmission of fascioliasis in animals in the Kashmir Valley. *British Veterinary Journal*, **145**: 57-61.

Silberstein, D.S. and Despommier, D.D. (1984). Antigens from *Trichinella spiralis* that induce a protective response in the mouse. *Journal of Immunology*, **132**: 898-904.

Silberstein, D.S. and Despommier, D.D (1985). Immunization with purified antigens protects mice from lethal infection with *Trichinella spiralis*. *Journal of Parasitology*, **71**: 516-517.

Sinclair, K.B. (1962). Observations on the clinical pathology of ovine fascioliasis. *British Veterinary Journal*, **118**: 37-53.

Sinclair, K.B. and Kendall, S.B. (1969). Precipitating antibodies to infection with *Fasciola hepatica* in rabbits. *Research in Veterinary Science*, **10**: 483-485.

Sinclair, K.B. (1970). The pathogenecity of *Fasciola hepatica* in previously infected and corticosteroid-treated lambs. *Research in Veterinary Science*, **11**: 209-215.

Sinclair, K.B. (1971a). Acquired resistance to *Fasciola hepatica* in sheep. *British Veterinary Journal*, **127**: 125-136.

Sinclair, K.B. (1971b). Resistance to *Fasciola hepatica* in sheep: attempts to transfer resistance with lymph node and spleen homogenate. *British Veterinary Journal*, **127**: 408-418.

Sinclair, K.B. (1973). The resistance of sheep to *Fasciola hepatica*. Studies on the development and pathogenicity of challenge infections. *British Veterinary Journal*, **129**: 236-250.

Sinclair, I.J and Joyner, L.P. (1974). The effect of the administration of a homologuous antigen on the establishment of *Fasciola hepatica* in the rabbit. *Research in Veterinary Science*, **16**: 320-327.

Sinclair, K.B. (1975). The resistance of sheep to *Fasciola hepatica*: studies in the pathophysiology of challenge infections. *Research in Veterinary Science*, **19**: 296-303.

Sloan, T., Dooge, D. and Joyce, P. (1991). Identification of phosphorylcholine containing antigens of *Fasciola hepatica*-successful tolerization against this epitope in experimental animals. *Parasite Immunology*, **13**: 447-455.

Smyth, J.D. (1962). Introduction to Animal Parasitology, 1st ed. The English University Press Ltd., London.

Solano, M. Ridley, R.K. and Minocha, H.C. (1991). Production and characterization of monoclonal antibodies against excretory-secretory products of *Fasciola hepatica. Veterinary Parasitology*, **40**: 227-239.

Soulsby, E.J.L. (1957). Some immunological phenomena in parasitic infections. *Veterinary Record*, **69**: 1129-1136.

Soulsby, E.J.L. (1982). *Heminths, Arthropods and Protozoa of Domesticated Animals*. 7th edition. Bailliere, Tindall and Cassel, London.

Sugane, K., Howell, M.J and Nichola, W.L. (1985). Biosynthetic labelling of excretory and secretory antigens of *Toxocara canis* larvae. *Journal of Helminthology*, **59**: 147-151.

Thorpe, E. and Broome, A.W.J. (1962). Immunity to *Fasciola hepatica* infection in Albino rats vaccinated with irradiated metacercariae. *Veterinary Record*, **74**: 755-756.

Thorpe, E. (1965). An immunocytochemical study with *Fasciola hepatica. Parasitology*, **55**: 209-214.

Threadgold, L.T. (1967). Electron microscope studies of *Fasciola hepatica*. III. Further observations on the tegument and associated structures. *Parasitology*, **57**: 633-637.

Threadgold, L.T. (1976). *Fasciola hepatica*: Ultrastructure and histochemistry of the glycocalyx of the tegument. *Experimental Parasitology*, **39**: 119-134.

Towbin, H. Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheet: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*, **76**: 4350-4354.

Urquhart, G.M. (1954). The rabbit as host in experimental fascioliasis. *Experimental Parasitology*, **3**: 38-44.

Urquhart, G.M., Armour, J., Duncan, J.L., Dunn, A.M. and Jennings, F.W. (1988). *Veterinary Parasitology*. pp 101. Longman Scientific and Technical, UK Ltd.

Urquhart, G.M., Mulligan, W. and Jennings, F.W. (1954). Artificial immunity to *Fasciola hepatica* in rabbits. 1. Some studies with protein antigens of *F. hepatica. The Journal of Infectious Diseases*, **94**: 126-133.

Urquhart, G.M., Jarrett, W.F.H. and Mulligan, W. (1962). Helminth immunity. *Advances in Veterinary Science*, **7**: 87-129.

Veira, L.Q., Gazzinelli, G., Kusel, J.R., Souza, C.P.S. DE and Colley, D.G. (1986). Inhibition of human peripheral blood mononuclear cell proliferative responses by released materials from *Schistosoma mansoni* cercariae. *Parasite Immunology*, **8**: 333-343.

Voller, A., Bidwell, D.E. and Barlett, A. (1979). *The Enzyme Linked Immunosorbent Assay (ELISA). A guide with abstracts of microplate applications*. Dynatech Europe, Borough House, Guernsey, UK, PP 1-42.

Warburg, O. and Christian, W. (1941). Isolierung ung kristallisation des garungsferments enolase. *Biochemische Zeitschrift*, **310**: 384 - 421.

Webster, C.C. and Wilson, P.N. (1980). Agriculture in the Tropics. In: '*Tropical Agriculture Series*', 2nd edition. Edited by D. Rhind. William Clowes and sons Ltd., London.

Wiest, P.M., Tisdale, E.J., Roberts, W.L., Rosenberry, T. L., Mahmoud, A.A.F and Tartakoff, A.M. (1988). Characterization of [3H]Palmitate- and [3H]ethanolamine-labelled proteins in the multicellular parasitic trematode *Schistosoma mansoni*. *Biochemical Journal*, **254**(2): 419-426.

Wilson, R.A. and Barnes, P.E. (1977). The formation and turnover of the membranocalyx on the tegument of *Schistosoma mansoni*. *Parasitology*, **74**: 61-71.

Wolowczuk, I., Auriault, C., Grass-Masse, H., Vendeville, J.M., Balloul, J.M., Tartar, A., and Capron, A. (1989). Protective immunity in mice vaccinated with the S. mansoni P28 - 1 antigen. *Journal of Immunology*, **142**: 1342.

Wolowczuk, I., Auriault, C., Bossus, M., Boulanger, D., Grass-Masse, H., Mazingue, C., Pierce, J.R., Grezel, D., Reid, D.G., Tartar, A. and Capron, A. (1991). Antigenicity and immunogenicity of a multiple peptidic construction of the *Schistosoma mansoni* Sm-28 GST antigen in the rat, mouse and monkey. *The Journal of Immunology*, **146**: 1987-1995.

Younis, S.A., Yagi, A.I., Haroun, E.M., Gameel, A.A., Taylor, M.G. (1986). Immunization of zebu calves against *Fasciola gigantica*, using irradiated metacercariae. *Journal of Helminthology*, **60**: 123-134.

Yoshihara, S., Taira, N. and Suzuki, K. (1981). Antigenic comparison among several developmental stages of *Fasciola* spp. *Japanese Journal of Veterinary Science*, **43**: 699-707.

Yoshihara, S., Nakagawa, M. and Suda, H. (1985). Protection against *Fasciola gigantica* infection in rats adminitered metacercarial antigens. *Research in Veterinary Science*, **39**: 383-384.

Zimmerman, G.L., Clark, C.R.B. (1986). Separation of parasite antigens by molecular exclusion, and chromatofocusing utilizing FPLC protein fractionation systems. *Veterinary Parasitology*, **20**: 217-228.

APPENDIX ONE: TABLES OF VALUES

Table A4.2 Fluke burdens of rats in trials to produce antiserum

No of flukes	No of rats Trials		
	1	2	3
0	7	7	-
1	15	13	3
2	5	8	5
3	3	5	9
4	3	2	10
5	3	2	2
6	1	-	-
7	-	1	1
8	-	-	2
10	-	-	1
11	-	-	1
14	-	-	1

Table A4.3 Fluke burdens of individual rats in the three passive protection trials

		Number of flukes in rats Trials		
Treatment				
	Dose	1	2	3
Antiserum (10 ml) at Days 0 and 2	20	0,6,4,5, 6,3,2 3	0,0,0,0,0, 0,0,1,1,2	0,0,1,1,1 1,1,1,1,2
Normal rat serum Days 0 and 2	20	1,2,2,6, 8,11,7	1,1,1,2,2 2,2,3,3	1,1,1,2 3 5,5,5,6
No serum	20	1,3,3,5, 8,9,11,6	0,0,0,1,2 2,2,4,6,8	1,3,3 3 4 4,5,4,5,6

Table A4.3.1(a) **Measurements (mm) of flukes recovered from rats in the passive protection trials**

Trial 1

Immune rat serum		Normal serum control		Challenge control	
L	B	L	B	L	B
12	5	9	5	16	8
12	6	12	6	14	8
13	7	15	8	15	7
16	8	13	7	12	6
8	4	9	6	13	7
16	9	12	6	10	5
12	6	13	6	13	6
10	5	12	7	10	5
13	6	10	5	10	5
11	6	14	7	13	7
14	7	17	8	13	6
15	7	18	9	16	8
9	5	13	7	6	3
12	6	8	5	10	5
17	8.5	9	5	9	5

Table A4.3.1(b) **Measurements (mm) of flukes recovered from rats in the passive protection trials**

Trial 2

Immunized		Normal serum control		Challenge control	
L	B	L	B	L	B
12	7	10	5	14	7
12	6	12	6	13	6
14	7	14	7	13	7
15	8	12	6	10	6
		8	5	11	5
		13	7	20	10
		11	6	19	10
		10	5	18	9
		14	8	10	5
		20	9	9	4
		13	7	10	6
		13	7	8	4
		17	8	15	7
		9	5	12	6
		11	6	11	5

Table A4.3.1(c) **Measurements (mm) of flukes recovered from rats in passive protection trial 3**

Trial 3					
Immune rat serum		Normal serum control		Challenge control	
L	B	L	B	L	B
20	9	14	5	8	3
18	8	12	5	16	8
13	6	20	10	18	9
18	9	13	5	15	7
17	8	12	5	19	9
11	6	17	8	10	4
16	8	16	6	8	4
20	9.5	14	7	12	6
14	6.5	15	7	18	8
		20	9	12	5.5
		16	9	12	5
		15	7	9	4.5
		19	8	17	9
		20	10	14	6.5
		18	9	17	9

Table A4.4.1(a) **OD values of obtained from titration of somatic extract of *F. hepatica* against 1:200 dilution of sera from rats with 1-3, 4-6 flukes and uninfected control rats in trial 1**

Concentration of extract (μg)	Mean OD values		
	Group		
	1-3 F (positive)	4-6F (positive)	Control (negative)
50	1.545	1.713	1.043
10	1.571	1.702	0.720
5	1.044	1.041	0.212
2	0.688	0.749	0.092
1	0.517	0.548	0.065
0.5	0.185	0.208	0.026
0.25	0.104	0.104	0.032
0.125	0.065	0.063	0.030

Table A4.4.1(b) **OD values following titration of known positive antisera and negative sera against somatic extract of adult fluke and enzyme conjugate**

Serum dilution	Mean OD values		
	Group		
	1-3F (positive)	4-6F (positive)	Control (negative)
1:50	0.513	0.438	0.189
1:100	0.375	0.304	0.115
1:200	0.315	0.268	0.090
1:400	0.257	0.176	0.091
1:800	0.231	0.144	0.050
1:1600	0.173	0.130	0.056
1:3200	0.113	0.098	0.050
1:6400	0.110	0.091	0.053
1:12800	0.111	0.075	0.045
1:25600	0.075	0.067	0.067
1:51200	0.070	0.065	0.051
1:102400	0.093	0.063	0.052

Table A4.4.1(c) **OD values following Titration of enzyme conjugate against somatic extract of adult fluke**

Conjugate dilution	Mean OD values		
	Group		
	1-3F (positive)	4-6F (positive)	Control (negative)
1:500	0.728	0.697	0.092
1:1000	0.619	0.581	0.068
1:2000	0.454	0.404	0.048
1:4000	0.277	0.218	0.031
1:8000	0.163	0.127	0.025
1:16000	0.093	0.079	0.027

Table A4.4.2(1a) ELISA response (mean OD) of trial 1 rats to adult ES

Group	D0	D14	D28	D42	D56	Correction factor	Negative control
	0.078	0.478	0.554	0.547	0.614		
	0.094	0.487	0.495	0.539	0.477		
	0.086	0.448	0.433	0.568	0.639		
	0.083	0.465	0.532	0.642	0.577	0.961	.074
	0.094	0.404	0.469	0.557	0.562		
	0.079	0.512	0.616	0.767	0.657		
	0.089	0.505	0.617	0.724	0.561		
	0.089	0.581	0.727	0.739	0.729		
	0.115	0.507	0.631	0.669	0.586		
	0.100	0.518	0.613	0.707	0.594		
1-3 F	0.091	0.538	0.606	0.698	0.658		
	0.093	0.538	0.593	0.685	0.654	0.964	.088
	0.090	0.420	0.472	0.631	0.482		
	0.094	0.548	0.569	0.641	0.548		
	0.097	0.567	0.567	0.660	0.624		
	0.096	0.520	0.610	0.622	0.543		
	0.087	0.474	0.537	0.621	0.552		
	0.088	0.419	0.510	0.563	0.511		
	0.090	0.547	0.615	0.671	0.671		
	0.093	0.510	0.591	0.690	0.714	0.897	.085
	0.094	0.521	0.574	0.682	0.709		
	0.095	0.512	0.577	0.691	0.629		
	0.093	0.514	0.607	0.665	0.647		
	0.065	0.515	0.748	0.762	0.708	0.897	.085
	0.103	0.607	0.652	0.751	0.630		
	0.103	0.660	0.763	0.749	0.722		
4-7 F	0.098	0.669	0.735	0.856	0.637	0.680	.095
	0.099	0.741	0.851	0.937	0.763		
	0.098	0.770	0.867	0.914	0.728		
	0.106	0.716	0.898	0.974	0.870		
	0.098	0.190	0.268	0.320	0.210		
	0.099	0.702	0.723	0.863	0.817	0.680	.095
0F+P	0.074	0.566	0.783	0.841	0.600		
	0.069	0.323	0.682	0.714	0.565		
	0.109	0.549	0.563	0.486	0.328	0.745	.064
0F-P	0.097	0.192	0.197	0.165	0.115		
	0.087	0.573	0.581	0.693	0.716		

Table A4.4.2(1b) Uninfected control rats

D0	D56	Correct factor	Negative control	D0	D56	Correction factor	Negative control
0.069	0.074	0.745	0.064	0.066	0.072	0.731	0.053
0.088	0.077			0.084	0.086		
0.091	0.085			0.082	0.085		
0.077	0.075			0.070	0.073		
0.068	0.081			0.069	0.071		
0.092	0.081			0.088	0.081		
0.070	0.068			0.073	0.070		
0.069	0.071	0.731	0.053	0.088	0.081		
0.073	0.070			0.086	0.089		
0.100	0.120			0.095	0.092		
0.100	0.087			0.095	0.092		
0.110	0.093			0.096	0.120		
0.098	0.102			0.102	0.106		
0.104	0.108			0.104	0.108		
0.094	0.095			0.101	0.112		
0.093	0.102			0.094	0.095		
0.097	0.091			0.095	0.097		
0.112	0.102			0.102	0.106		
0.098	0.095			0.104	0.104		
0.097	0.094			0.105	0.109		

Table A4.4.2(1.1a) ELISA response (mean OD) of trial 1 rats to somatic extract of adult fluke

Group	D0	D14	D28	D42	D56	Correction Factor	Negative control
1-3 F	0.088	0.515	0.637	0.815	0.651	1.00	0.085
	0.115	0.397	0.305	0.462	0.784		
	0.091	0.504	0.531	0.676	0.660		
	0.087	0.540	0.540	0.563	0.423		
	0.088	0.494	0.605	0.662	0.571		
	0.099	0.532	0.676	0.825	0.664		
	0.094	0.558	0.683	0.717	0.658		
	0.101	0.576	0.516	0.541	0.500		
	0.088	0.707	0.783	0.835	0.781	0.772	0.084
	0.095	0.666	0.708	0.766	0.832		
	0.093	0.682	0.691	0.809	0.826		
	0.095	0.717	0.674	0.874	0.826		
	0.086	0.520	0.601	0.706	0.718		
	0.086	0.552	0.611	0.645	0.590		
	0.090	0.592	0.675	0.623	0.619		
	0.094	0.493	0.493	0.742	0.542		
	0.074	0.758	0.751	0.796	0.909	0.932	0.056
	0.064	0.712	0.770	0.835	0.480		
	0.074	0.401	0.509	0.738	0.588		
	0.060	0.645	0.632	0.667	0.766		
	0.067	0.537	0.544	0.571	0.645		
	0.057	0.469	0.473	0.658	0.545		
	0.063	0.562	0.554	0.530	0.618		
	0.083	0.556	0.634	0.768	0.743	0.932	0.056
4-6 F	0.110	0.344	0.575	0.746	0.346	1.055	0.108
	0.090	0.502	0.505	0.732	0.643		
	0.110	0.453	0.529	0.667	0.514		
	0.111	0.369	0.570	0.737	0.626		
	0.111	0.262	0.311	0.675	0.554		
	0.108	0.360	0.414	0.615	0.729		
	0.109	0.152	0.176	0.191	0.153	1.055	0.108
	0.114	0.576	0.516	0.541	0.149		
0F+P	0.087	0.282	0.694	0.686	0.710	0.837	0.085
	0.089	0.591	0.734	0.745	0.452		
	0.090	0.409	0.475	0.543	0.569		
0F-P	0.090	0.580	0.631	0.700	0.607	0.837	0.085
	0.091	0.209	0.134	0.144	0.129		

Table A4.4.2(1.1b) Uninfected control rats

D0	D56	Correction factor	Negative control	D0	D56	Correction factor	Negative control
0.092	0.087	0.837	0.085	0.086	0.101	1.038	0.075
0.096	0.101			0.082	0.082		
0.104	0.089			0.079	0.102		
0.092	0.102			0.080	0.078		
0.092	0.081			0.082	0.076		
0.096	0.106			0.078	0.099		
0.087	0.088			0.081	0.080		
0.069	0.071	0.837	0.085	0.078	0.086		
0.073	0.070			0.077	0.089		
0.100	0.120			0.078	0.080		
0.100	0.087			0.080	0.095		
0.110	0.093			0.096	0.081		
0.098	0.102			0.077	0.077		
0.079	0.083	1.038	0.075	0.094	0.096		
0.079	0.082			0.080	0.091		
0.081	0.078			0.100	0.102		
0.078	0.090			0.069	0.075		
0.082	0.087			0.085	0.081		
0.087	0.087			0.094	0.083		
0.078	0.098			0.072	0.084		

Table A4.4.2(2a) ELISA response (mean OD) of trial 2 rats to ES of adult fluke

	D0	D14	D28	D42	D56	Correction Factor	Negative control
1-3 F	0.097	0.524	0.703	0.643	0.541	0.721	0.095
	0.086	0.509	0.607	0.655	0.624		
	0.084	0.623	0.710	0.748	0.798		
	0.085	0.556	0.563	0.696	0.728		
	0.096	0.617	0.672	0.811	0.893		
	0.097	0.515	0.503	0.587	0.683		
	0.104	0.507	0.643	0.661	0.755		
	0.105	0.537	0.607	0.684	0.511		
	0.126	0.486	0.621	0.854	0.925	0.790	0.090
	0.109	0.404	0.525	0.776	0.752		
	0.106	0.258	0.562	0.478	0.571		
	0.102	0.402	0.508	0.601	0.409		
	0.087	0.423	0.603	0.622	0.591		
	0.093	0.270	0.640	0.496	0.567		
	0.107	0.443	0.621	0.706	0.794		
	0.093	0.514	0.603	0.671	0.606		
	0.091	0.357	0.343	0.471	0.434	1.042	0.089
	0.091	0.316	0.392	0.449	0.408		
	0.093	0.348	0.411	0.499	0.298		
	0.094	0.372	0.423	0.491	0.425		
	0.090	0.515	0.539	0.621	0.705		
	0.094	0.388	0.448	0.548	0.440		
	0.100	0.379	0.473	0.510	0.544		
	0.103	0.457	0.463	0.505	0.554		
	0.094	0.339	0.519	0.633	0.642	1.087	0.098
	0.102	0.367	0.515	0.588	0.613		
4-6 F	0.097	0.343	0.435	0.534	0.541	1.087	0.098
	0.091	0.357	0.402	0.558	0.591		
	0.091	0.291	0.382	0.609	0.631		
	0.092	0.407	0.485	0.609	0.707		
	0.110	0.366	0.459	0.619	0.727		
0F+P	0.097	0.183	0.357	0.353	0.330	1.251	0.085
	0.100	0.189	0.199	0.434	0.367		
	0.100	0.335	0.363	0.350	0.295		
0F-P	0.099	0.187	0.309	0.260	0.282	1.251	0.085
	0.106	0.190	0.234	0.188	0.200		
	0.092	0.131	0.199	0.171	0.174		
	0.105	0.221	0.412	0.423	0.399		

Table A4.4.2(2b) Uninfected control rats

D0	D56	Correction factor	Negative Control	D0	D56	Correction factor	Negative control
0.101	0.096	1.231	0.093	0.089	0.090	1.008	0.075
0.096	0.099		0.087	0.077			
0.099	0.096		0.076	0.102			
0.101	0.098		0.081	0.067			
0.078	0.066		0.073	0.086			
0.097	0.100		0.078	0.094			
0.086	0.081		0.082	0.080			
0.077	0.065		0.088	0.086			
0.085	0.078		0.077	0.079			
0.078	0.092		0.069	0.080			
0.099	0.104		0.090	0.095			
0.102	0.100		0.079	0.084			
0.078	0.102		0.088	0.090			
0.082	0.086		0.066	0.096			
0.084	0.083		0.090	0.091			
0.091	0.092		0.100	0.102			
0.088	0.078		0.091	0.088			
0.088	0.089		0.075	0.082			
0.087	0.104		0.094	0.085			
0.101	0.098		0.064	0.092			

Table A4.4.2(2.1a) ELISA response (mean OD) of trial 2 rats to somatic extract of adult fluke

Group	D0	D14	D28	D42	D56	Correction Factor	Negative control
1-3 F	0.083	0.316	0.460	0.465	0.472	1.180	0.067
	0.070	0.344	0.460	0.479	0.510		
	0.096	0.308	0.397	0.460	0.490		
	0.070	0.410	0.489	0.520	0.479		
	0.070	0.347	0.445	0.476	0.485		
	0.071	0.278	0.380	0.473	0.412		
	0.076	0.500	0.530	0.555	0.496		
	0.082	0.306	0.414	0.429	0.453		
	0.090	0.351	0.466	0.471	0.487	1.255	0.076
	0.090	0.373	0.435	0.477	0.488		
	0.082	0.290	0.476	0.478	0.486		
	0.081	0.346	0.467	0.473	0.486		
	0.088	0.296	0.400	0.425	0.455		
	0.086	0.306	0.477	0.506	0.518		
	0.087	0.346	0.473	0.467	0.483		
	0.080	0.350	0.486	0.477	0.481		
	0.085	0.247	0.314	0.442	0.465	1.200	0.062
	0.093	0.285	0.320	0.371	0.435		
	0.086	0.348	0.460	0.489	0.483		
	0.087	0.315	0.471	0.466	0.491		
	0.088	0.346	0.458	0.476	0.486		
	0.095	0.381	0.460	0.489	0.499		
	0.088	0.342	0.461	0.469	0.472		
	0.094	0.255	0.335	0.380	0.411		
	0.079	0.311	0.371	0.412	0.460	0.987	0.075
	0.078	0.295	0.415	0.455	0.478		
4-6 F	0.065	0.184	0.308	0.533	0.409	1.320	0.062
	0.067	0.227	0.305	0.421	0.382		
	0.073	0.190	0.269	0.518	0.507		
	0.073	0.294	0.366	0.578	0.511		
	0.068	0.289	0.358	0.619	0.467		
0F+P	0.080	0.213	0.224	0.334	0.284	1.260	0.078
	0.080	0.204	0.237	0.278	0.258		
	0.088	0.177	0.200	0.327	0.291		
0F-P	0.090	0.158	0.307	0.279	0.254	1.260	0.078
	0.080	0.261	0.306	0.229	0.241		
	0.082	0.130	0.203	0.198	0.197		
	0.101	0.187	0.280	0.296	0.310		

Table A4.4.2(2.1b) Uninfected control rats

D0	D56	Correction Factor	Negative Control	D0	D56	Correction factor	Negative Control
0.078	0.066	0.837	0.085	0.068	0.080	1.038	0.075
0.072	0.068			0.086	0.082		
0.092	0.093			0.089	0.102		
0.078	0.088			0.078	0.079		
0.090	0.078			0.085	0.077		
0.086	0.076			0.088	0.099		
0.100	0.100			0.071	0.069		
0.101	0.071	0.837	0.085	0.098	0.086		
0.097	0.092			0.069	0.089		
0.078	0.067			0.090	0.084		
0.089	0.087			0.091	0.092		
0.097	0.094			0.085	0.082		
0.088	0.082			0.078	0.079		
0.089	0.083	1.038	0.075	0.080	0.100		
0.089	0.085			0.078	0.087		
0.078	0.088			0.088	0.087		
0.075	0.087			0.078	0.079		
0.089	0.090			0.080	0.081		
0.077	0.087			0.079	0.082		
0.079	0.090			0.067	0.078		

Table A4.4.2(3a) ELISA response (mean OD) of rats in trial 3 rats ES of adult fluke

Group	D0	D14	D28	D42	D56	Correction factor	Negative control
	0 062	0.153	0.183	0.183	0.207		
	0.069	0.147	0.154	0.212	0.199		
	0.081	0.144	0.146	0.173	0.210		
	0.089	0.168	0.250	0.234	0.212	3.624	.074
	0.065	0.131	0.166	0.191	0.188		
	0.063	0.070	0.109	0.156	0.182		
	0.067	0.063	0.092	0.165	0.194		
	0.065	0.098	0.150	0.168	0.107		
1-3 F	0.048	0.135	0.155	0.196	0.171		
	0.045	0.090	0.164	0.138	0.144		
	0.046	0.091	0.142	0.142	0.150		
	0.048	0.097	0.122	0.126	0.200	4.500	.088
	0.045	0.070	0.110	0.171	0.189		
	0.048	0.067	0.120	0.196	0.212		
	0.046	0.077	0.129	0.115	0.123		
	0.094	0.083	0.121	0.106	0.123		
	0.047	0.143	0.210	0.320	0.365		
	0.059	0.108	0.166	0.202	0.270		
	0.061	0.132	0.164	0.232	0.247		
	0.058	0.098	0.173	0.205	0.226	4.037	.085
	0.058	0.091	0.130	0.229	0.205		
	0.061	0.087	0.146	0.291	0.235		
	0.064	0.121	0.143	0.252	0.265		
>4 F	0.084	0.091	0.166	0.230	0.257		
	0.081	0.179	0.235	0.290	0.285		
	0.077	0.109	0.166	0.295	0.282		
	0.082	0.088	0.192	0.207	0.255		
	0.078	0.218	0.213	0.303	0.278		
	0.114	0.139	0.159	0.302	0.176	2.590	0.095
	0.150	0.141	0.158	0.244	0.165		
	0.095	0.141	0.195	0.234	0.193		
	0.076	0.170	0.239	0.307	0.234		
	0.104	0.144	0.160	0.247	0.179	2.3	0.073
	0.078	0.172	0.240	0.307	0.234		

Table 4.4.2(3b) Uninfected control rats

D0	D56	Correction factor	Negative control	D0	D56	Correction factor	Negative control
0.074	0.072	0.709	0.075	0.066	0.080	1.018	0.073
0.071	0.066			0.078	0.073		
0.082	0.084			0.082	0.064		
0.068	0.078			0.069	0.073		
0.080	0.068			0.072	0.077		
0.076	0.066			0.068	0.070		
0.100	0.090			0.067	0.065		
0.101	0.071	0.817	0.065	0.071	0.075		
0.097	0.092			0.070	0.072		
0.073	0.065			0.070	0.080		
0.081	0.083			0.081	0.082		
0.078	0.074			0.066	0.069		
0.088	0.082			0.050	0.059		
0.084	0.078	1.018	0.073	0.060	0.073		
0.077	0.075			0.072	0.077		
0.078	0.088			0.067	0.065		
0.075	0.069			0.064	0.065		
0.089	0.075			0.073	0.081		
0.078	0.084			0.072	0.082		
0.079	0.090			0.067	0.068		

Table A4.4.2(3.1a) ELISA response (mean OD) of trial 3 rats to somatic extract of adult fluke

Group	D0	D14	D28	D42	D56	Correction factor	Negative control
	0 079	0.228	0.313	0.365	0.644		
	0.104	0.275	0.293	0.385	0.351		
	0.101	0.292	0.344	0.459	0.398		
	0.074	0.261	0.395	0.548	0.426	2.540	.074
	0.098	0.244	0.257	0.343	0.264		
	0.109	0.163	0.313	0.315	0.370		
	0.078	0.127	0.197	0.288	0.298		
	0.086	0.188	0.274	0.330	0.379		
	0.098	0.196	0.384	0.568	0.383		
	0.097	0.189	0.361	0.307	0.372		
	0.101	0.139	0.280	0.309	0.368		
1-3 F	0.098	0.129	0.232	0.213	0.184	2.900	.088
	0.097	0.150	0.211	0.369	0.228		
	0.098	0.176	0.275	0.390	0.355		
	0.097	0.138	0.195	0.255	0.170		
	0.106	0.119	0.190	0.257	0.207		
	0.092	0.183	0.300	0.376	0.213		
	0.093	0.168	0.296	0.313	0.306		
	0.100	0.209	0.295	0.393	0.342		
	0.104	0.242	0.270	0.391	0.329	2.800	.085
>4 F	0.104	0.210	0.247	0.335	0.300		
	0.098	0.197	0.280	0.425	0.322		
	0.100	0.164	0.268	0.447	0.331		
	0.097	0.134	0.294	0.367	0.252		
	0.093	0.213	0.380	0.310	0.483		
	0.095	0.124	0.229	0.313	0.400		
	0.088	0.103	0.291	0.430	0.252		
	0.090	0.220	0.336	0.578	0.417		
	0.089	0.129	0.201	0.514	0.435	2.560	0.095
	0.090	0.136	0.120	0.250	0.220		
	0.090	0.136	0.227	0.344	0.270		
	0.100	0.196	0.197	0.307	0.399		
	0.101	0.136	0.095	0.251	0.229		
	0.088	0.197	0.197	0.312	0.299		

Table A4.4.2(3.1b) Unifected control rats

D0	D56	Correction factor	Negative control	D0	D56	Correction factor	Negative control
0.084	0.076	0.917	0.075	0.084	0.101	1.008	0.065
0.067	0.101			0.078	0.080		
0.078	0.085			0.079	0.089		
0.067	0.078			0.075	0.076		
0.078	0.088			0.084	0.081		
0.087	0.087			0.088	0.090		
0.084	0.083			0.071	0.080		
0.078	0.074			0.067	0.076		
0.078	0.084			0.087	0.088		
0.100	0.101			0.068	0.066		
0.095	0.087			0.069	0.095		
0.094	0.093			0.086	0.081		
0.094	0.092			0.097	0.077		
0.083	0.074			0.084	0.106		
0.073	0.082			0.063	0.091		
0.082	0.074			0.084	0.092		
0.074	0.090			0.073	0.075		
0.075	0.086			0.080	0.081		
0.078	0.067			0.094	0.083		
0.065	0.067			0.072	0.084		

Table A4.4.3(1a) ELISA response (mean OD) of passive protection trial 1 rats to ES of adult fluke

Group	D0	D14	D28	D42	D56	Correction Factor	Negative control
	0.072	0.573	0.385	0.234	0.120		
	0.082	0.570	0.658	0.639	0.332		
	0.095	0.561	0.526	0.595	0.517		
+IRS	0.077	0.504	0.602	0.715	0.531	1.134	0.075
	0.084	0.506	0.606	0.645	0.741		
	0.080	0.470	0.504	0.669	0.480		
	0.081	0.432	0.504	0.405	0.341		
	0.083	0.432	0.466	0.643	0.394		
	0.079	0.157	0.204	0.246	0.158		
	0.80	0.146	0.170	0.209	0.190		
	0.082	0.247	0.335	0.316	0.345		
+NRS	0.089	0.229	0.307	0.457	0.283	1.611	0.078
	0.085	0.305	0.328	0.434	0.414		
	0.084	0.291	0.407	0.410	0.422		
	0.084	0.250	0.301	0.330	0.329		
	0.081	0.182	0.241	0.548	0.424		
	0.073	0.183	0.241	0.457	0.284		
	0.077	0.202	0.252	0.398	0.344		
NS	0.083	0.237	0.246	0.457	0.376	1.604	0.069
	0.089	0.229	0.296	0.482	0.379		
	0.074	0.232	0.262	0.399	0.380		
	0.081	0.245	0.328	0.448	0.414		
	0.070	0.261	0.323	0.518	0.431		

Table A4.4.3(1b) ELISA response (mean OD) of passive protection trial 1 rats to somatic extract of adult fluke

Group	D0	D14	D28	D42	D56	Correction Factor	Negative control
	0.109	0.365	0.280	0.202	0.189		
	0.085	0.600	0.590	0.649	0.574		
	0.075	0.551	0.564	0.674	0.568		
+IRS	0.083	0.574	0.670	0.768	0.638	1.070	0.069
	0.109	0.546	0.550	0.618	0.600		
	0.101	0.603	0.672	0.803	0.556		
	0.117	0.469	0.508	0.648	0.500		
	0.072	0.484	0.540	0.678	0.546		
	0.076	0.269	0.386	0.602	0.574		
	0.084	0.366	0.551	0.668	0.575		
	0.096	0.293	0.421	0.587	0.546		
+NRS	0.087	0.316	0.408	0.552	0.500	1.020	0.065
	0.071	0.293	0.446	0.616	0.551		
	0.078	0.337	0.43	0.588	0.543		
	0.101	0.213	0.196	0.185	0.175		
	0.092	0.306	0.438	0.774	0.700		
	0.096	0.393	0.548	0.838	0.708		
	0.098	0.316	0.396	0.628	0.610		
NS	0.103	0.494	0.623	0.888	0.698	1.040	0.088
	0.089	0.268	0.348	0.538	0.518		
	0.092	0.288	0.401	0.653	0.550		
	0.098	0.290	0.425	0.601	0.549		
	0.090	0.286	0.334	0.605	0.538		

Table A4.4.3(2a) ELISA response (mean OD) of passive protection trial rats to ES of adult fluke

Group	D0	D1	D3	D14	D28	D42	D56	Correction Factor
+IRS	0.095	0.551	0.563	0.465	0.293	0.195	0.100	
	0.099	0.439	0.536	0.422	0.428	0.379	0.335	.884
	0.091	0.517	0.577	0.482	0.309	0.133	0.095	
	0.103	0.607	0.599	0.588	0.431	0.258	0.219	
	0.095	0.523	0.592	0.429	0.431	0.201	0.206	
	0.092	0.590	0.643	0.465	0.244	0.209	0.095	.898
	0.096	0.511	0.577	0.358	0.345	0.154	0.166	
	0.091	0.555	0.607	0.391	0.431	0.257	0.363	
	0.101	0.628	0.670	0.477	0.263	0.159	0.116	.872
	0.090	0.660	0.694	0.521	0.277	0.218	0.184	
+NRS	0.096	0.092	0.130	0.475	0.476	0.502	0.339	
	0.095	0.155	0.172	0.466	0.455	0.597	0.540	.872
	0.100	0.130	0.183	0.415	0.485	0.589	0.474	
	0.097	0.138	0.160	0.385	0.451	0.481	0.397	.919
	0.101	0.105	0.154	0.170	0.194	0.216	0.230	
	0.098	0.144	0.145	0.462	0.503	0.618	0.680	
	0.106	0.152	0.212	0.340	0.434	0.483	0.485	
	0.103	0.099	0.127	0.459	0.506	0.592	0.580	1.04
	0.099	0.149	0.158	0.367	0.373	0.490	0.533	
NS	0.104	0.097	0.100	0.467	0.563	0.515	0.575	
	0.172	0.112	0.099	0.272	0.258	0.344	0.293	1.05
	0.090	0.089	0.101	0.525	0.634	0.650	0.640	
	0.090	0.096	0.102	0.324	0.441	0.448	0.435	
	0.094	0.100	0.095	0.384	0.432	0.428	0.398	
	0.095	0.078	0.094	0.355	0.432	0.431	0.378	1.05
	0.094	0.069	0.088	0.376	0.459	0.530	0.486	
	0.099	0.090	0.089	0.333	0.429	0.570	0.530	
	0.097	0.088	0.065	0.417	0.465	0.555	0.565	1.05
	0.099	0.069	0.077	0.145	0.164	0.117	0.102	

Table A4.4.3(2b) ELISA response (mean OD) of passive protection trial 2 rats to somatic extracts of adult fluke

Group	D0	D1	D3	D14	D28	D42	D56	Correction Factor
+IRS	0.018	0.485	0.610	0.450	0.413	0.281	0.241	
	0.007	0.600	0.630	0.460	0.400	0.204	0.201	1.2
	0.090	0.532	0.500	0.406	0.340	0.199	0.192	
	0.006	0.495	0.540	0.326	0.306	0.174	0.121	
	0.010	0.567	0.580	0.350	0.296	0.166	0.141	
	0.084	0.532	0.618	0.391	0.256	0.234	0.222	850
	0.676	0.567	0.642	0.430	0.259	0.213	0.186	
	0.090	0.532	0.583	0.413	0.264	0.208	0.193	
	0.089	0.536	0.628	0.424	0.285	0.258	0.184	.938
	0.094	0.544	0.603	0.373	0.266	0.223	0.179	
+NRS	0.085	0.103	0.098	0.476	0.563	0.650	0.622	
	0.079	0.084	0.085	0.404	0.464	0.642	0.554	.986
	0.100	0.094	0.077	0.461	0.484	0.586	0.521	
	0.094	0.074	0.083	0.354	0.358	0.496	0.440	
	0.066	0.068	0.086	0.452	0.479	0.563	0.530	
	0.074	0.077	0.092	0.312	0.389	0.466	0.435	.720
	0.088	0.092	0.080	0.359	0.544	0.678	0.588	
	0.078	0.083	0.074	0.394	0.401	0.537	0.520	
	0.090	0.113	0.105	0.309	0.391	0.578	0.302	1.30
NS	0.096	0.103	0.095	0.296	0.280	0.230	0.200	
	0.089	0.093	0.088	0.545	0.630	0.710	0.840	1.25
	0.101	0.077	0.096	0.530	0.608	0.696	0.769	
	0.096	0.113	0.098	0.501	0.602	0.789	0.820	
	0.097	0.099	0.116	0.496	0.585	0.660	0.740	
	0.098	0.097	0.101	0.037	0.240	0.210	0.190	1.04
	0.094	0.088	0.078	0.486	0.545	0.630	0.680	
	0.099	0.108	0.110	0.487	0.570	0.664	0.700	
	0.112	0.099	0.103	0.360	0.190	0.185	0.180	1.02

Table A4.4.3(3a) ELISA response (mean OD) of passive protection trial 3 rats to to ES product adult fluke

Group	D0	D1	D3	D14	D28	D42	D56	Correction Factor
+IRS	0.090	0.145	0.168	0.073	0.093	0.093	0.071	
	0.109	0.118	0.126	0.068	0.073	0.092	0.095	3.5
	0.099	0.160	0.182	0.100	0.106	0.150	0.127	
	0.104	0.180	0.190	0.098	0.108	0.148	0.130	
	0.105	0.190	0.210	0.131	0.135	0.180	0.159	
	0.107	0.210	0.250	0.150	0.135	0.191	0.160	3.56
	0.100	0.201	0.205	0.160	0.136	0.200	0.159	
	0.102	0.203	0.208	0.132	0.122	0.174	0.131	
	0.074	0.200	0.220	0.118	0.115	0.113	0.115	3.88
	0.086	0.182	0.209	0.077	0.110	0.036	0.046	
+NRS	0.093	0.100	0.094	0.093	0.122	0.208	0.229	
	0.102	0.099	0.102	0.107	0.154	0.176	0.142	3.88
	0.093	0.087	0.110	0.089	0.100	0.183	0.133	
	0.093	0.095	0.096	0.107	0.117	0.190	0.138	4.1
	0.099	0.097	0.101	0.115	0.136	0.200	0.112	
	0.094	0.095	0.095	0.132	0.174	0.215	0.145	
	0.097	0.100	0.099	0.106	0.151	0.215	0.241	
	0.095	0.093	0.092	0.139	0.178	0.206	0.200	3.94
	0.094	0.097	0.100	0.095	0.110	0.152	0.132	
NS	0.101	0.099	0.096	0.209	0.269	0.221	0.214	
	0.097	0.097	0.101	0.240	0.296	0.320	0.259	3.9
	0.080	0.085	0.088	0.215	0.197	0.230	0.210	
	0.099	0.096	0.095	0.136	0.140	0.212	0.211	
	0.098	0.093	0.099	0.076	0.081	0.101	0.101	
	0.086	0.088	0.098	0.142	0.184	0.224	0.210	4.05
	0.102	0.098	0.092	0.145	0.223	0.213	0.215	
	0.094	0.088	0.099	0.141	0.148	0.219	0.205	
	0.083	0.090	0.099	0.145	0.223	0.213	0.215	4.10

Table A4.4.3(3b) ELISA response (mean OD) of passive protection trial 3 rats to somatic extract of adult fluke

Group	D0	D1	D3	D14	D28	D42	D56	Correction Factor
	0.093	0.366	0.279	0.157	0.156	0.123	0.112	
	0.088	0.325	0.272	0.136	0.148	0.153	0.168	2.54
	0.089	0.354	0.297	0.202	0.166	0.210	0.149	
	0.117	0.317	0.314	0.231	0.192	0.325	0.260	
+IRS	0.109	0.258	0.245	0.161	0.122	0.139	0.151	
	0.104	0.206	0.197	0.157	0.157	0.165	0.144	4.00
	0.108	0.178	0.207	0.142	0.126	0.192	0.153	
	0.098	0.173	0.173	0.156	0.153	0.195	0.159	
	0.077	0.356	0.321	0.261	0.267	0.352	0.348	3.98
	0.070	0.289	0.543	0.152	0.225	0.057	0.069	
	0.085	0.092	0.082	0.097	0.126	0.286	0.363	
	0.101	0.094	0.099	0.142	0.203	0.219	0.207	2.88
	0.092	0.088	0.101	0.107	0.138	0.273	0.226	
+NRS	0.089	0.088	0.092	0.115	0.145	0.303	0.219	2.9
	0.079	0.089	0.094	0.120	0.145	0.24	0.352	
	0.091	0.090	0.091	0.128	0.181	0.42	0.270	
	0.086	0.090	0.090	0.111	0.221	0.374	0.439	
	0.071	0.092	0.087	0.136	0.192	0.422	0.278	2.78
	0.092	0.093	0.101	0.108	0.137	0.272	0.227	
	0.084	0.085	0.094	0.184	0.155	0.439	0.507	
	0.100	0.102	0.096	0.171	0.206	0.278	0.447	2.96
	0.099	0.091	0.097	0.124	0.165	0.317	0.409	
	0.102	0.100	0.102	0.144	0.242	0.376	0.408	
NS								
	0.101	0.094	0.095	0.075	0.081	0.158	0.082	
	0.101	0.094	0.080	0.135	0.217	0.315	0.315	2.80
	0.093	0.089	0.092	0.154	0.242	0.345	0.342	
	0.097	0.089	0.095	0.163	0.289	0.570	0.548	
	0.090	0.093	0.099	0.183	0.155	0.439	0.507	2.84

Table A4.5.1(a) **Mean percentage eosinophil in trial 3 rats**

	Day after infection				
Group	0	14	28	42	56
	0	0	18	0	0
	0	0	0	0	0
	0	0	6	6	3
	0	0	1	0	0
+IRS	0	0	20	16	3
	0	1	15	9	5
	0	0	0	6	2
	0	3	9	14	15
	0	0	0	0	2
	0	0	11	3	2
	0	0	14	7	8
	0	0	13	3	7
	0	14	3	9	2
	0	1	21	18	12
+NRS	0	2	17	9	6
	0	1	20	12	2
	0	4	9	10	14
	0	3	9	6	16
	0	2	14	3	2
	0	1	17	9	17
	0	1	22	7	12
	0	2	13	0	2
	0	1	15	2	8
NS	0	1	2	1	0
	0	1	18	8	5
	0	1	17	6	2
	0	4	13	6	4
	0	1	25	1	10

Table A4.5.1(b) Mean PCV of rats from trial 3

	Day after infection				
Group	0	14	28	42	56
	40	51	55	50	50
	48	60	54	62	55
	40	59	57	50	53
	52	54	53	45	56
+IRS	46	55	50	49	58
	42	52	47	50	47
	47	50	50	45	42
	46	51	53	49	50
	49	45	54	48	56
	49	57	40	45	48
	40	44	53	42	43
	45	54	55	59	52
	50	55	52	50	52
	43	59	56	59	55
+NRS	44	46	46	44	46
	50	60	49	52	52
	44	52	58	50	50
	51	55	54	64	50
	46	50	50	52	45
	40	52	50	46	48
	50	52	52	44	38
	44	52	52	42	48
	46	50	52	48	52
NS	56	59	54	55	56
	58	56	58	54	48
	45	56	50	44	40
	44	48	55	60	58
	40	50	58	54	56

Table A4.5.1(c) Mean percentage of neutrophils of rats from trial 3

	Day after infection				
Group	0	14	28	42	56
	30	30	31	29	25
	35	28	25	23	19
	38	16	10	25	63
	40	22	6	39	29
+IRS	40	38	14	40	14
	30	35	26	28	20
	30	20	29	44	22
	24	28	31	35	30
	27	19	20	30	23
	18	24	25	38	18
	40	34	35	51	17
	26	68	34	44	37
	28	35	35	50	15
	40	40	30	39	32
+NRS	21	64	52	41	15
	30	50	39	37	30
	22	29	32	45	20
	24	50	49	49	48
	27	40	45	55	27
	30	40	46	38	36
	32	47	49	34	36
	18	29	35	45	24
NS	27	25	42	43	19
	25	31	18	18	14
	24	33	24	65	23
	33	36	29	51	19
	41	41	33	43	27
	28	37	40	52	21

Table A4.5.1(d) Mean percentage lymphocytes in rats from trial 3

	Day after infection				
Group	0	14	28	42	56
	70	83	54	35	74
	67	76	73	76	66
	65	76	71	57	33
	66	74	78	35	66
+IRS	64	55	56	40	83
	60	57	49	38	71
	69	73	56	46	70
	77	69	65	66	74
	63	55	59	32	74
	75	74	44	72	64
	76	70	55	53	79
	74	56	37	34	70
	73	28	54	42	48
	60	54	38	33	48
+NRS	65	51	40	24	82
	68	32	23	43	50
	72	43	35	37	74
	77	68	52	38	61
	50	42	26	39	54
	62	55	48	42	59
	68	40	20	41	48
	75	61	43	49	71
	77	58	30	46	71
NS	69	54	72	75	83
	66	55	39	19	68
	76	48	46	39	73
	58	47	46	48	59
	60	47	27	40	59

Table A4.5.1(e) Mean percentage of monocytes in rats from trial 3

	Day after infection				
Group	0	14	28	42	56
	8	4	3	7	6
	13	8	2	1	14
	12	10	13	12	1
	11	4	9	12	3
+IRS	7	9	9	4	4
	15	8	10	5	4
	13	5	7	9	7
	11	6	10	15	0
	7	3	1	4	0
	6	12	8	7	1
	13	8	12	7	5
	7	4	9	11	8
	6	9	6	8	1
	15	8	9	19	6
+NRS	14	4	7	7	7
	8	6	6	14	12
	14	3	6	7	5
	13	4	17	6	5
	10	5	13	14	7
	8	8	12	8	2
	7	7	9	18	3
	13	6	17	6	3
	14	13	12	9	2
	15	13	8	6	3
NS	8	7	18	8	4
	14	14	7	4	6
	10	9	7	3	10
	4	3	8	7	9

APPENDIX TWO PREPARATION OF SOLUTIONS AND REAGENTS

ALGAL CULTURE

Solution A	g/L
Potassium nitrate	8.0
Potassium dihydrogen orthophosphate	10.32
Sodium Chloride	0.16
Sodium Carbonate	0.08
Ferrous sulphate	0.08

Solution B	mg/100 ml
Ammonium vanadate	2
Boric acid	286
Cobalt nitrate	5
Copper sulphate	8
Magnesium sulphate	25
Manganous chloride	180
Sodium molybdate	20
Zinc sulphate	2

Solution C	
Calcium chloride	5.5g/100 ml

To 250 ml of solution A add 900 ml of distilled water followed by 1.0 ml of solution B and 1.0 ml of solution C, and make up to 1 L (for 1 kg of soil sample).

PBS-PI-METHIONINE:

(200 ml solution)	
TPCK	10 mg
TLCK	5 mg
Methionine	20 mg
PMSF	34.8 mg in 1 ml ethanol.

COMPLETE CULTURE MEDIUM

RPMI 1640	180 ml
Add by filtration:	
Penicillin	100 units
Streptomycin	100 µg
I mM L-Glutamine	2 ml
ß-Mecarptoehanol	0.761 µg
Foetal Calf serum (sterile)	20 ml
Fungizone (no filtration)	100 µg

COMPLETE MEDIUM FOR BIOSYNTHETIC LABELLING

1. Dispense 250 ml of RPMI 1640 (Selectamine, Gibco) into a flask containing 250 ml of sterile, deionized distilled water.

2. Reconstitute each amino acid, (excepting the one to be used for labelling) with 10 ml of sterile, deionized distilled water and add to the medium.

3. Reconstitute the vitamin mixture with 10 ml of sterile deionized water and add to the medium.

4. Add 20 ml of 10% glucose and 10 ml of 0.05% phenol.

5. Add 27 ml of 7.5% $NaCO_3$ to the medium.

6. Adjust pH to 7.0 with 1N HCl or NaOH.

7. Bring volume to 1 L.

8 Aliquot and store in 20ml volumes at -70°C.

FINAL PREPARATION OF MEDIUM FOR BIOSYNTHETIC LABELLING

1. Defrost 20 ml of medium, and pipette off 600 µl.

2. Add through millipore filtration.

penicillin	200 units
Streptomycin	200 µg
1 mM L-Glutamine	200 µl
Foetal Calf Serum	600 µl
Fungizone	20 µg

SOLUTIONS FOR ELISA

Borate buffered saline (pH 8.2)

Boric acid	6.18 g
Di-sodium tetraborate	9.54 g
Sodium chloride	4.38 g
Distilled water to 1 L	

Washing solution

Nacl	72 g
Tween 20	4 g
Distilled water to 8 L	

Sodium acetate/citrate buffer

Solution A: 49.2 g of sodium acetate (anhydrous) made up to 1 L with distilled water (0.6 M). Solution B: 52.5 g of citric acid made up to 500 ml with distilled water (0.5 M). x% stock was prepared by adding 100 ml of solution B to 50 ml of solution A and adjusting pH to 6.0. Stock was diluted 5x for use.

TMB substrate

3,3',5,5'-Tetramethylbenzidine (TMB)	5 mg
(Dissolved in 0.5 ml DMSO [analar])	
0.1 M Sodium acetate/citric acid buffer	
(pH 6.0)	50 µl
Hydrogen peroxide (30% w/v)	7.5 µl

SDS-PAGE SOLUTIONS

Solution A

1 M HCL	96 ml
Tris	73.2g
TEMED	92 µl

Made up to 200 ml with distilled water. (pH 6.7)

Solution B

Acrylamide	150 g
Bis-acrylamide	4 g

Made up to 500 ml with distilled water.
Add 33.33 g of BAD-Duolite MB 6113 mixed resin and stir for 1-2 h to remove excess acrylic acid.

Stacking buffer

Tris	17.94 g
1 M HCL	144 ml

Bring up to 300 ml with distilled water (pH 6.7)

Preparation of 10-20% gradient solutions for large gels

	10%	20%
Solution A	3.12 ml	3.12 ml
Solution B	8.32 ml	16.64 ml
Distilled water	2.48	-
10% SDS	250 µl	250 µl
Solution D	10.79 ml**	4.95 ml*

** = 50 mg ammonium persulphate in 100 ml H_20.
* = 74.94 mg ammonium persulphate in 100 ml H_20.

Stacking gel

Stacking buffer	5 ml
Solution B	4 ml
TEMED	50 µl
10% SDS	400 µl
Distilled water	33.55 ml
10% ammonium persulphate	250 µl

Preparation of 12% separating solution for mini gels

1.5 M Tris-HCL	3.35 ml
10% SDS	2.5 ml
Solution B	4 ml
10% ammonium persulphate	50 µl
TEMED	5 µl

Stacking gel

Distilled water	6.1 ml
0.5 M Tris-HCL	2.5 ml
10% SDS	100 µl
Solution B	1.3 ml
10% ammonium persulphate	50 µl
TEMED	10 µl

WESTERN BLOTTING

Blocking buffer: pH 7.4

Tris	12.1 g
NaCl	17.4 g
EDTA	0.74 g
Gelatin	5 g
NP 40	1 ml

Distilled water to 2 L

Bjerrum and Schafer-Nielsen Transfer buffer

Tris	11.64 g
Glycine	5.86 g
SDS	0.0075 g
Methanol	400 ml

Adjust volume to 2 L with double distilled water

CO-PRECIPITATION DILUENT

Buffers

1. 2.0M Tris/HCl (i.e, 121 g Tris in 500 ml of distilled H_20, pH 8)
2. 10% NP40
3. 0.5 M NaCL

Diluent

1.	2.0M Tris/Hcl pH 8	10 ml
2.	0.5M NaCl	100 ml
3.	10% NP 40	20 ml

Make up to 1L, pH 8.0.

SOLUBILIZING BUFFERS

Solution 1

Trisma base	45 mg
EDTA	223 mg

(made up to 10 ml in distilled water)

Solution 2

SDS	1 mg
ßME	0.5 g
Glycerol	5 ml

Made up to 10 ml in distilled water. Add bromophenol blue to colour

Solution 3

SDS	1 g
Iodoacetamide	0.5 g
Glycerol	5 ml

Made up to 10 ml in distilled water. Add pouceau-S to colour

Reducing buffer and non-reducing buffers

Add equal volumes of solution 1 and 2 to prepare reducing buffer and equal volumes of solutions 1 and 3 to rpepare non-reducing buffer.

APPENDIX THREE MANUFACTURERS' NAMES AND ADDRESSES

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Hawkley (Gelman Hawskley & Sons)
10 Harrowden
Breckmills
NORTHHAMPTON

Hereaus Equipment Ltd
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CM15 9TB

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